UNIVERSIDAD AUTÓNOMA DE MADRID
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Alteraciones histológicas y mecanismos de fibrosis en las lesiones varicosas de la vena safena interna: Estudio inmunohistoquímico, molecular y de microscopía confocal.

TESIS DOCTORAL

Doctorado en Medicina y Cirugía

Juan Pedro Velasco Martín

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JAVIER FRANCISCO REGADERA GONZÁLEZ, MÉDICO PATÓLOGO Y CATEDRÁTICO DE HISTOLOGÍA HUMANA DE LA FACULTAD DE MEDICINA DE LA UNIVERSIDAD AUTÓNOMA DE MADRID y

GABRIEL ESPAÑA CAPARRÓS, JEFE DE SERVICIO DE CIRUGÍA VASCULAR DEL HOSPITAL MONCLOA, UNIVERSIDAD EUROPEA DE MADRID

CERTIFICAN QUE: D. **JUAN PEDRO VELASCO MARTÍN,** Licenciado en Biología por la Universidad Autónoma de Madrid, ha realizado bajo nuestra dirección el trabajo de investigación **"Alteraciones histológica y mecanismos de fibrosis en las lesiones varicosas de la vena safena interna: estudio inmunohistoquímico, molecular y de microscopía confocal."**, estudio que consideramos completamente satisfactorio para ser presentado y defendido como Tesis Doctoral en el Programa de Doctorado de Medicina y Cirugía de la Universidad Autónoma de Madrid.

Lo que firmamos en Madrid el 7 de septiembre de 2015

Fdo.: Javier Regadera González
Director

Fdo.: Gabriel España Caparrós
Director

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INTRODUCTION

Varicose veins (tortuous, twisted, or lengthened veins[123] is a common vascular disease of the lower extremity. Varices are sometimes classified into trunk, reticular and hyphenweb types based on their size and anatomical distribution.[75] Chronic Venous Insufficiency (CVI) is a common problem with a significant impact on both afflicted individuals and the health care system.[73] In this sense, varicose veins affecting more than 25 million adults in the United States, and more than 6 million suffering more advanced venous disease.[27]

Venous pathology develops when venous pressure is increased and return of blood is impaired through several mechanisms.[41] The overall increased venous pressure as a result of blood stasis may result in vein wall dilatation and the characteristic CVI dermal changes with hyperpigmentation, venous eczema, subcutaneous tissue fibrosis and ultimately ulceration.[72,225] One general functional consequence of varicose vein formation and/or venous insufficiency is a decrease in venous return and an increase in the filling pressure of the associated venous network – a phenomenon that is referred to as 'venous hypertension'.

Although many risk factors have been proposed, there is still some controversy about the etiology and pathogenesis of the varicose disease.[77] The prevalence of varicose veins is higher in developed, industrial countries than in underdeveloped countries. Risk factors found to be associated with CVI include age, sex, and family history of varicose veins, obesity, pregnancy, phlebitis and previous leg injury.[120,150,248] There are also environmental or behavioral factors associated with CVI, such as prolonged standing and perhaps a sitting posture at work.[120,145] Like we had said, varicose vein disease is a frequently occurring pathology with multifactorial causes and a genetic component,[239] as is corroborated by the frequent clinical observation that the distensibility of arm veins in patients with varicose veins is increased abnormally, suggesting a systemic disease of the venous wall.[306]

Many clinical studies have shown that the prevalence of varicose veins is approximately twice as high in women as men, and increases with advancing age.[49,75,150,253] The Edinburgh Vein Study screened 1566 subjects with duplex ultrasound for reflux, finding CVI in 9.4% of men and 6.6% of women after age adjustment, which rose significantly with age (21% in men over 50 years and 12% in women over 50 years).[233] Also, the Framingham

Study demonstrated a prevalence of 1 % in men and 10 % in women aged less than 30 years, compared with 57 per cent in men and 77 per cent in women over 70 years old.[34]

Varicose veins are more common in women who have had several pregnancies and had had hemorrhoids and vulvar varicosities during and after pregnancy.[100] The development of new varicose veins occurs in up to 28 % of pregnancies.[258] During pregnancy, weight gain from increased total bodyfluid and raised intra-abdominal pressure may predispose varicose vein formation in woman. Furthermore, upregulation of certain hormones, such as relaxin, oestrogen and progesterone, causes venous relaxation and increases vein capacitance.[199]

Family history is another described risk factor. A study in France reported that a history of varicose veins in a first-degree relative is the most important risk factor in both men and women.[46] Patients with varicose veins were 21.5 times more likely to report a positive family history.[248] Another study in Japan found that 42 % of patients with varicose veins had a positive family history compared with only 14 % in those without varicosities.[105] In conclusion, epidemiological studies have demonstrated an involvement of hereditary factors for the transmission of varicose veins.[61,81] Fiebig A. *et al.* 2010[78] conclude that the additive genetic component of CVI is approximately 17 %.

Finally, the Tampere study interviewed 3,284 randomly chosen men and 3,590 women 40 to 60 years of age.[150] The questionnaire covered family status, sex, age, profession and weight. With these parameters it was possible to correlate the incidence of varicose veins with specific life style factors. From the data one can conclude that varicose veins correlate with female sex, obesity, extensive standing type of work, parity (proportional to the number of given births) and a family history of varicose disease. The latter relation points to a possible genetic predisposition to develop varicose veins. Interestingly, most risk factors, such as Body Mass Index, parity and prolonged standing, lead to a rise in hydrostatic pressure and may therefore augment venous remodeling.[216]

CLINICAL, ETIOLOGY, ANATOMIC, PATHOPHYSIOLOGY (CEAP) CLASSIFICATION OF CHRONIC VENOUS INSUFFICIENCY

The CEAP classification was introduced in 1996, defining clinical (C), etiological (E), anatomical (A) and pathophysiological (P) aspects of CVI. The manifestations of CVI may be viewed in terms of a well-established clinical classification scheme. The CEAP classification was developed by an international consensus conference to provide a basis of uniformity in reporting, diagnosing, and treating CVI (Table 1).[218] The clinical classification has 7 categories (0–6) and is further categorized by the presence or absence of symptoms. The classification is a valuable tool in the objective evaluation of CVI, providing a system to standardize CVI classification with emphasis on the manifestations, cause, and distribution of the venous disease.[249] It has become widely accepted as the standard classification system for venous disorders.[131] And the CEAP classification shows a statistically significant association between a higher CEAP grade and an older current age of patients.[46]

CEAP CLASSIFICATION VENOUS DISEASE
CLINICAL CLASSIFICATION (C)
0 (No visible or palpable signs of venous disease)
1 (Telangiectasies or reticular veins)
2 (Varicose veins)
3 (Edema)
4 (Pigmentation or eczema)
5 (Healed venous ulcer)
6 (Active venous ulcer)
ETIOLOGICAL CLASSIFICATION (E)
Congenital (Present at birth or develop in childhood)=Ec
Primary (Develop independent of other diseases)=Ep
Secondary (Develop as a consequence of another pathology)=Es
ANATOMICAL CLASSIFICATION (A)
Superficial veins (The great and small saphenous veins)=As
Deep veins (Cava, popliteal, crural, tibial, femoral, etc)=Ad
Perforating veins (Connect superficial veins with deep veins)=Ap
PATHOPHYSIOLOGY (P)
Reflux (Retrograde flow)=Pr
Obstruction (Venous obstruction)=Po
Reflux and obstruction (Both)=Pro

Table 1. Basic CEAP Classification.

PATHOPHYSIOLOGY OF VEIN WALL ALTERATIONS IN VARICOSE DISEASE OF LEG

A normal venous system depends on the integrity of the vein valves, vein wall structure and the hemodynamics of venous blood flow. These components are interdependent and the disruption of one affects the integrity of the others.[164] The primary cause of varicose vein formation is not clear; however, both vein valve dysfunction and hydrostatic venous pressure appear to play a critical role in the initiation and progression of the disease.[225] Venous valvular incompetence and elevation in the pressure of blood in the

lower-limb superficial venous system is considered contributory to vein dilatation and tortuosity sees in varicose veins.[73]

Two principal theories, the so-called valvular hypothesis[172,173] and the vein wall hypothesis,[232] have been put forward to explain the pathogenesis of varicose veins and chronic venous insufficiency (CVI). One hypothesis proposes that valvular dysfunction causing reflux is the initial pathological change that occurs in varicose veins. Venous reflux then causes blood stasis and venous hypertension, which damages the vein wall leading to weakness and dilatation. Venous dilatation separates the valve cusps further and worsens the valvular incompetence, triggering a vicious cycle. Varicose veins also demonstrate hypotrophy of the valves and widening of the valvular annulus compared with non-varicose veins.[59,60,207]

The theory of primary venous dilatation leading to secondary valvular incompetence has received more attention nowadays. This plausible hypothesis has been challenged recently by several common ultrasonography and histological findings relating to varicose veins. Varicosities are often observed below competent valves, and not uncommonly found to precede valvular incompetence. Venous dilatation is also frequently seen distal to a valve rather than proximal, which one would expect if valvular dysfunction is the initial event.[143,199,232] It has been reported that varicose veins can develop without valvular incompetence.[58,297] Although valve reflux may precede vein-dilatation,[246] there is a significant body of evidence supporting the view that vein dilation can precede venous reflux, and that valvular dysfunction may be an epiphenomenon of vein wall dilation.[84,130,225]

Also, findings from duplex ultrasonography on the pattern and progression of venous dilatation and reflux strongly support vein wall changes as the primary event, although isolated primary valvular dysfunction may still sometimes contribute. The valves of varicose veins contain less collagen and lose the normal viscoelastic features typical of non-varices.[221] Monocyte and macrophage infiltration into the valvular sinuses is also greater than that in the distal vein wall of varicose veins, indicating increased inflammatory activity in the valves.[207] The accord to this theory, distal valves may also become incompetent secondary to the proximal reflux and dilatation, leading to a retrograde progression of disease.[186,225,255]

Several stresses related to blood stasis and venous hypertension, including hypoxia, mechanical stretch and low shear stress, have been postulated to contribute to vein wall changes.[190,199] As well as, Elsharawy MA. *et al.* 2007[74] supported the theory of primary weakness of the vein wall as a cause of varicosity. They said that this weakness is due to intimal changes, disturbance in the connective tissue components and smooth muscle cells. Their study has shown that there was no significant difference in the vein wall structural changes between varicose veins with and without valve incompetence, supporting the theory of primary weakness in the vein wall leading to dilatation of the vein with resultant separation of valve cusps.[93]

Although incompetent perforators have also been implicated as a cause of primary varicose veins, the correlation of incompetent perforators with varicose veins on pathological and thermographic studies has been poor. Several studies demonstrate that incompetent perforator veins are larger in diameter than competent ones,[66,261,302] suggesting that, as with other primary varicose veins, perforator reflux develops secondarily from a primary problem with vein wall integrity. Once established, perforator reflux contributes to superficial venous hypertension, especially in the setting of concomitant deep system venous reflux. Anyway, it seems clear that varicose veins do not always start at the saphenofemoral or saphenopopliteal junction and progress in a descending manner. Anterograde disease progression is more likely to be caused by primary vein wall changes leading to subsequent valvular incompetence than vice versa. Alternatively, the primary valvular incompetence may represent a multicentric process that develops simultaneously in discontinuous venous segments.[144,199]

VENOUS ANATOMY AND BLOOD FLOW IN THE LEG

Anatomy studies of lower limbs demonstrated that peripheral venous system is divided into superficial and deep venous systems. Superficial veins are located outside the muscle-fascial compartments and are connected to the deep venous system by perforating veins. The perforating veins traverse the anatomic fascial layer to connect the superficial to the deep venous system.[73] There are two major truncal veins in the superficial system, the great and small saphenous veins. The venous system functions as a blood capacitance

reservoir and also a channel to return the blood to the heart. As veins are exposed to lower pressure, it is logical to expect less mechanical stretch and shear stress in veins in comparison to arteries. In the erect position, blood that enters into the lower extremity venous system must travel against gravity and other pressures to return to the central circulation and prevent retrograde flow into the legs called venous reflux).[73] There is a series of 1-way bicuspid valves located throughout the deep and superficial veins that open to allow flow toward the heart but close to prevent the return of blood toward the feet.[174]

The valves function in concert with venous muscle pumps to allow the return of blood against gravity to the heart.[209] Contraction of the muscle pumps, primarily in the calf, force blood out of the venous plexus to ascend up the deep venous system. The valves prevent blood from being forced more distally within the deep system or through perforator veins into the superficial system.[73] In addition, perforating veins also contain valves that only allow blood flow from the superficial to the deep veins.[73] From the clinical perspective, the peripheral venous system is important, because leads to increased pooling of blood in the legs and high venous pressure exerting a static stretch on the vein wall.[72] Fail of this system is associated with chronic venous insufficiency (CVI).

Blood flow in varicose veins is disrupted (Figure 1), resulting in blood stasis and reflux. The cause and sequence of events leading to such inefficient blood flow remain unclear, although valvular incompetence, vein wall weakness and venous dilatation are features associated with varicose veins.[164]

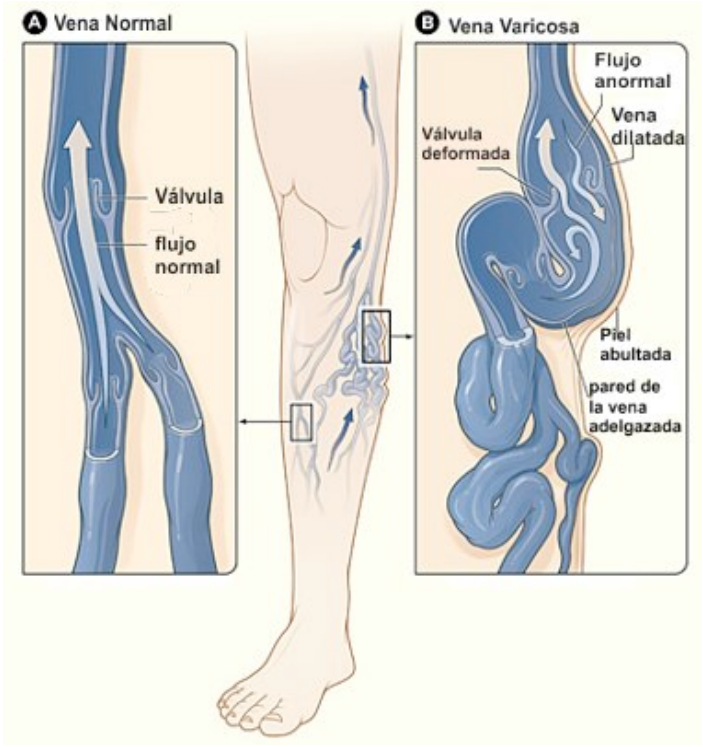

Figure 1. Diagram of blood flow at a normal vein and at a varicose vein.

Like we had said, the anatomic classification of the peripheral venous system describes the superficial, deep, and perforating venous systems, with multiple venous segments that may be involved. The failure in the venous circulation can be localized to superficial, deep, or perforating veins of the lower extremities.[27] There are various causes of varicose veins in the lower extremities.

HISTOLOGY, IMMUNOHISTOCHEMISTRY AND MOLECULAR BIOLOGY OF VEIN

Veins are histological and functionally organized in three layers: intima, media and adventitia (Figure 2). The different layers of the vascular wall exert their own influence on both the vasomotor control and the vascular structure, being the final effect the result of the interrelated participation of the three layers. Endothelial, smooth muscle cells (SMCs) and different immunocompetent cell types embedded in the extracellular matrix (ECM) are observed in saphenous vein wall.

The ***tunica intima*** delimits the vessel wall towards the lumen of the vessel and comprises the endothelium, consisting of a simple layer of epithelium, the basal lamina and

the intimal connective tissue (Figure 2). Under the basement membrane, normal vessels contain a layer of elastic fibers, called the internal elastic lamina, composed of SMCs separated by interlaminar matrix collagens, microfibrils, proteoglycans, glycoproteins, and ground substance.[287] Although the media components can be different between different types of blood vessels. Veins, for example, have less collagens and elastin than arteries have.

The endothelium is not only a mechanical barrier but also acts as receptor and transmitter of signals between blood and other components of the vascular wall. Endothelial cells (ECs) have exocrine, paracrine and autocrine functions, and they are involved in the regulation of vascular tone, vasculogenesis and angiogenesis, blood coagulation, fibrinolysis, and inflammation.[188] ECs are sensitive to hemodynamic changes such as pressure or shear stress forces and to circulating chemical messengers. ECs respond to these signals by secreting different growth factors and vasoactive substances, including vasodilator factors such as NO and prostaglandin (PG) I2 or prostacyclin (PGI2) which also inhibits platelet aggregation.[83] Main vasoconstrictor factors released from the endothelium are endothelin-1 (ET-1), Reactive Oxygen Species (ROS) and vasoconstrictor prostanoids such as thromboxane A2 (TXA2) and PGE2.[83,270]

The ***tunica media*** is formed by a layer of circumferentially arranged vascular SMCs and variable amounts of ECM. The tunica media is separated from the tunica adventitia by a second layer of elastic fibers, the external elastic lamina, which is located above the vascular SMCs (Figure 2). Elastic fibers are found throughout the vessel wall in the medial layer, where they arrange in concentric fenestrated elastic laminae. Vascular SMCs can release a variety of vasoconstrictor and vasodilator substances including prostanoids and ROS, among others.[155] The tunica media respond to the action of different vasoactive factors and hemodynamic forces by contracting or dilating the vessels thus being essential in the control of vascular tone.

The ***tunica adventitia*** is mainly composed of connective tissue, fibroblasts, immunocompetent cells and perivascular adipose tissue (Figure 2). Depending on the vessel type, a number of small arteries termed *vasa vasorum* can be found to facilitate blood irrigation to the vessel wall. Adventitia also receives the neuronal axons that innervate the

muscular tissue.[229] In the last years it has become evident that the adventitia is not only a mechanical support for the vessel but also has an active role in the regulation of vascular tone and structure[259] by releasing different factors such as free fatty acids, adipokines,[208] adipose-derived relaxing factor,[169] ROS[104,228] and COX-2 derived prostanoids.[7,28]

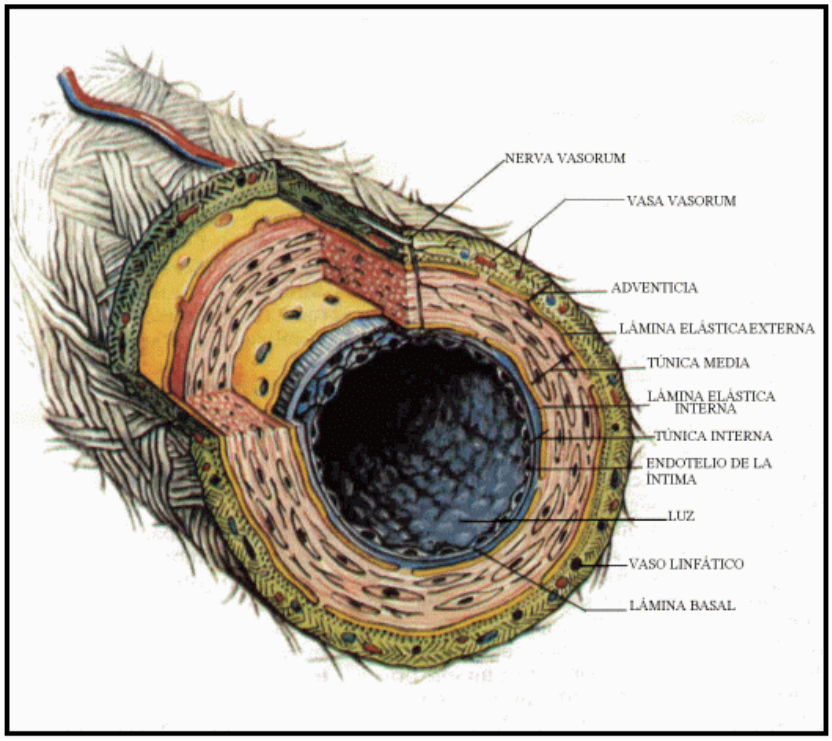

Figure 2. General structure of the vessel wall.

Extracellular matrix in vascular wall

The ECM is an important structural and functional scaffolding made up of proteins (including collagen, elastin, fibronectin, growth factors, proteoglycans and glycosaminoglycans).[36] These molecules are produced by ECs, SMCs and adventitial cells and are necessary for a variety of cell functions, including cell differentiation and signaling, cellular migration, angiogenesis, blood vessel support, epithelialization, and wound repair.[36,129,287] In this sense, the ECM can release a repertoire of insoluble ligands that induce

cell signaling to control proliferation, migration, differentiation and survival.[36] The same ECM proteins at different regions of the blood vessel wall may come from different cells and be regulated by different modulators under certain circumstances. In the media, collagen and elastin are produced primarily by SMCs. In the adventitia, however, ECM proteins -such as collagen, osteopontin, and fibronectin- primarily come from fibroblasts, as in other connective tissues.[299]

Collagen is a very stiff protein that has the physiological role to limit vessel distension.[36] The polypeptide precursor of the collagen molecules, procollagen, is secreted to the extracellular compartment where it transforms in tropocollagen. After enzymatic modification, the mature collagen monomers aggregate and become crosslinked to form collagen fibers. The predominant vascular collagens are type I collagen and type III collagen, which comprise up to 80-90% of the total blood vessel wall collagens (Type I collagen about 60% and type III about 30% of the total collagen.[114] Type IV collagen is a major component of the basal lamina of blood vessels, which plays an important role in regulating pro- and anti-angiogenic events.[175]

Elastin is the major protein that imparts the property of elasticity to blood vessels. Elastin functions as a cross-linked polymer as part of an elastic fiber and its assembly outside the cell requires an association with numerous other extracellular proteins such as microfibrils.[15,36,287] Elastin represents 90% of the elastic fibers. Elastin is normally produced by SMCs in the media and by fibroblasts in the adventitia. Elastin deposition in normal vascular wall is limited to the media layer extending from the internal to external elastin laminae. Under normal conditions, elastogenesis is restricted mainly to fetal life and infancy, and mature elastic fibers last for the entire lifespan. The half-life of elastin fibers is about 40 years; elastic fibers are considered the most durable element of ECM.[15] Elastic fibers are degraded and fragmented with age and disease, leading to increased stiffness of the vessel wall.[98] Under pathological conditions, vascular (SMCs, ECs, and fibroblasts) make elastin as part of the reaction to increased mechanical stress.[126]

The precursor of elastin, tropoelastin, is a highly hydrophobic protein, which is soluble in salt solution. Mature elastin fibrils are insoluble and intertwined with collagen, fibrilin and

fibulin proteins, and also with carbohydrates.[213,286] Elastin comprises repetitive sequences and multiple chains cross-linked by desmosine linkages formed among lysine residues modified by lysyl oxidase.[213,286] Although collagen and elastin are the major contributors to the visco-elastic characteristics of the vascular wall, a number of additional ECM components affect both the compliance characteristics and vaso regulatory abilities of blood vessels.

Fibronectin is a multi-domain ECM protein that interacts with multiple integrins, heparan sulfate proteoglycans, collagens, and fibrins to mediate cellular behaviors.[211] The content of fibronectin in blood vessels is important because it modifies the mean stress and elastic modulus of the wall.[197] Fibronectin is produced and secreted by numerous cell types including SMCs, fibroblasts and myofibroblasts, and is widely distributed in ECM. Fibronectin function in the vasculature is mediated by α5β1 integrin, which is expressed by ECs, SMCs, and fibroblasts, and is widely distributed in ECM.[299] In addition, fibronectin controls the deposition, organization, and stability of other matrix molecules including type I collagen, type III collagen and thrombospondin-1, as well as, fibronectin modulates leukocyte infiltration, expression of adhesion molecules, cell proliferation, and vascular SMCs phenotypic differentiation. All factors involved in vascular remodeling processes.[55]

Integrins are transmembrane proteins that mediate attachment of the cell to the ECM and have a role in signal transduction from the ECM to the cell.[86] Integrins represent the largest family of cell surface receptors. It has been suggested that changes in integrin profile are important for processes leading to more chronic structural rearrangement of the vascular wall and ECM material.[197]

Matrix metalloproteinases (MMPs) are a family of endopeptidases which require a zinc ion at their active site for proteolytic activity.[47] These enzymes are able to degrade most constituents of the ECM.[225] They are mainly responsible for the degradation and reorganization of ECM that can lead to physiological or pathological processes. Thus, in normal physiological vascular remodeling, MMP activity is tightly controlled at different levels. However, factors that promotes vessel remodeling upregulate MMP activities. Loss of control of MMP activity could result in degradation of ECM, enabling vascular SMCs to

migrate and proliferate, as well as inflammatory cells to infiltrate the vessel wall.[31,47] Thus, partial degradation of the ECM surrounding vascular SMCs is likely a necessary step for allowing repositioning of cells during remodeling.[197] MMPs are inactivated by endogenous tissue inhibitors TIMPs. So, homoeostasis of the ECM is regulated by MMPs and TIMPs.[108,165,225,255]

***Plasminogen/plasminogen activator system*.** Plasmin can degrade ECM directly or indirectly via MMP activation.[31] Therefore altered plasmin activity can have high impact on vascular fibrosis. Plasmin is released as a zymogen called plasminogen. The activity of this system is tightly controlled by plasminogen activator inhibitor type 1 that functions as the principal inhibitor of tissue plasminogen activator.

Tenascins are ECM glycoproteins. Tenascin C is mainly found in vessels and it was the first member of a family of four structurally similar proteins identified including tenascin R, W and X.[92] Tenascin C has diverse functions including weakening of cell adhesion, up-regulation of the expression and activity of MMPs, modulation of inflammatory responses, promotion of myofibroblasts recruitment and enhancement of fibrosis.[110]

CELLULAR AND MOLECULAR PATHOLOGY OF VARICOSE VEIN

Like we have said, recent studies of varicose vein pathogenesis have focused on the structural and biochemical changes in the vein wall.[186,225,255] Zsoter T. *et al.* 1966[306] has suggested that veins from patients with varicosities are more distensible than those from patients with normal veins, indicating a probable systemic basis for the abnormality.

It is well known that vessel wall remodeling occurs as an adaptation to pressure and flow (e.g., vein graft) or to mechanical (e.g., angioplasty) or biochemical (e.g., atherosclerosis) injuries, all of which promote ECM-regulated SMCs migration and proliferation.[202] Evidence has shown that the tension generated by intraluminal pressure affects the thickness and composition of the vessel wall.[147] Mechanical stretch exerted by increased intraluminal pressure induces vascular smooth muscle hypertrophy and hyperplasia and changes in contractile and matrix proteins.[157] Localised haemodynamic stresses in vessels are considered to play a role in attracting inflammatory cells into the

vessel wall.[4]

Areas of intimal hyperplasia with associated collagen deposits, SMC infiltration, subendothelial fibrosis and luminal dilation are common in varicose veins.[74,231,255,290] Changes in the media, including SMC proliferation, ECM degradation, fragmentation of elastic lamellae and loss of circular and longitudinal muscle fibers are seen more often in varicose than in non-varicose veins.[19,74,289] Similarly, the adventitia of varicose veins demonstrates areas of increased SMCs, fibroblasts, and connective tissue with others regions of atrophy and devoid of *vasa vasora*.[19,199] Also, focal aggregates of macrophages were described within the media and adventitia of both normal and varicose veins.[298] The low-magnification microscopic appearance of a varicose vein section consists of a thickened vein wall with disruption of the normal organization of the extracellular matrix (ECM) and SMCs.[199] Previous studies[221,242,276] shown that there were significant changes in collagen, elastin and SMCs contents of the wall of varicose veins compared to the normal, even without saphenofemoral valve incompetence.

The media of varicose veins exhibit areas of SMC hypertrophy and proliferation, but also regions of atrophy are also present.[170,199,255,276,289,290] Rearrangement and migration of SMCs into the intima may also be seen.[74,255,290] Some studies have reported an increase in amounts of SMCs or their activity in varicose veins,[220] whereas, others found reduced amounts of SMCs due to replacement by connective tissue. [19,232]

The ECM is a dynamic structure that maintains the integrity and homoeostasis of the vein through interactions with cellular components such as the endothelium and SMCs.[108] Cellular interaction with ECM regulates cell adhesion, migration, proliferation, phenotype, and tissue architecture under different circumstances.[299] ECM remodeling, precede the progression of varicosities. Its degradation is likely to contribute to the weakening and dilatation of veins. ECM, containing mainly elastin and collagen, is essential for vessel homeostasis and contributes to the strength, flexibility, and structural integrity of the vascular wall.[162] Degradation of ECM is mainly caused by an array of proteolytic enzymes, including MMPs and serine proteases, which are produced by both vascular ECs and fibroblasts cells as well as white blood cells,[178,179] in particular during inflammation.[109,192]

Disruption of the elastic fibers, including fragmentation of the elastic laminas, and thickening of individual collagen fibers have often been observed in varicose veins.[219,289,290]

The overall collagen content in varicose compared with non-varicose veins remains unclear, with some studies reporting an increase,[84,241,289,290] but some describing no change[284] and some showing a reduction.[8] Additionally, the subtypes of collagen in varicose veins are different from those in non-varicose veins. It has been shown that type I collagen is significantly increased in segments of varicose veins compared with control saphenous veins.[288] Also, Kirsch D. *et al.* 2000[128] have shown that there is significant increase in matrix proteins such as type IV collagen and laminin in the wall of varicose veins compared with normal veins. Moreover, a dysregulation in collagen synthesis is described in varicose veins.[240,242] Immunohistological and Transmission electron microscopy results showed alterations in the distribution pattern of type I, III, and IV collagen in the wall of varicose veins compared with control saphenous veins.[90] Ghaderian & Khodaii tagged the immunohistochemical pattern of type I collagen revealed strong staining in the subendothelial region of most varicose vein specimens as did control saphenous vein specimens which is an indication of an increase of this type of collagen in the subendothelial region.[90]

Indeed, others investigators have quantified the overproduction of SMCs derived from varicose veins of type I collagen, which provides tensile strength, decreased production of type III collagen, which contributes to elasticity, and similar quantities of type V collagen in varicose veins compared with control saphenous veins.[242,243,288] This imbalance might have consequences for the mechanical properties of the tissue. Moreover, the imbalance in the synthesis of type I collagen and type III collagen can affect vein wall function in varicose veins as described in “the weak wall hypothesis”. However, their observations were based on collagen fibers derived from cultures of varicose vein SMCs do not explain the distribution of collagen types in the three layers of veins.

Alteration of SMCs behavior from quiescent or “contractile state” typical of the normal vessel phenotype to a proliferative or “synthetic state” characteristic of the varicose phenotype increases collagen synthesis.[9] These synthetic SMCs synthesize larger amounts of

the ECM components and lose the expression of the contractile filaments leading to the thickening of the venous wall and loss of the contractility in the varicose vein.[189] However, others believe there is a reduction in the cellularity of the smooth muscle layer with replacement by collagen or a significant increase in collagen content of varicose veins.[184] Anyway, development of vascular stiffening, at least in arterial hypertension, has always been linked to excessive deposition of collagen in the vessel walls.[111,206]

Elastin, the other main protein of the ECM in vessel, acts as more than a venous wall structural protein for the storage of recoiling energy. In vitro, many cells exhibit migration and proliferation in response to tropoelastin, elastin degradation products, and elastin peptides.[299] Development of varicose veins therefore involves elastin component reconstruction, even when elastin expression is turned off in adults;[39] however, an increase of elastin is found in arterial an d venous diseases. In vivo, elastic fibers and laminae prohibited SMC proliferation and prevented intimal hyperplasia.[158] In a rat model of adventitial implantation of collagen, basal lamina, and elastic laminae patches, elastic laminae patches, but not laminin or collagen patches, are associated with reduced neointima formation and SMC proliferation.[167] In fact, mature elastin fibers are key elements in the maintenance of acquiescent vascular SMC phenotype by providing a physical barrier for cellular migration.[299]

During the development of varicose veins, the elastic network is damaged and deregulated, showing reduced and fragmented elastin along with disordered collagen distribution.[3,241] At damaged during aging or tissue injury, elastic fibers are generally not replaced, because elastin expression is turned off in adults. Instead, more collagens are made, shifting the arterial wall toward a stiff arrange of collagen fibers.[299] Other authors marked that varicose veins show high latent TGF-β) binding protein (LTBP)-2 and TGFβ expression, particularly in the subendothelium and media, and in areas with marked injury. However, the intimal mechanisms implicated in molecular alterations of varicose vein are not well established and several studies about gene expression were done. It is showed the upregulated genes include those of ECM molecules, cytoskeletal proteins and myofibroblasts such as transforming growth factorβ-induced gene (BIGH3), tubulin, lumican, actinin, type I

collagen, versican, actin and tropomyosin.[154]

In primary varicose veins compared to normal veins, some authors demonstrated a reduction in collagen and elastin content.[74,284] In contrast, some have found an increase in the collagen content without change[276] or reduction[84,242] in elastin content in segments of varicose veins compared to normal. It seems probable that alteration in the balance of elastin and collagen content is likely to contribute to varicose vein wall weakening.[74,289,290] The diffusely disorganised architecture and distribution of collagen and elastin fibres corresponds to fibrotic degradation of the parietal wall and a loss of mechanical properties in varicose veins.[40,134] Therefore, the pathological abnormalities in varicose veins were not due to deficiency of smooth muscles layers, but could be referred to the inability of SMCs to provide the necessary tone in the vessel wall leading to vein wall dilatation.[232] This SMC dysfunction[38] may be due to the break-up of its regular arrangement by fibrous tissue[289] as effective contraction cannot occur when individual cells are not in direct communication with each other.[232] Previously it is referred that the formation of varicose veins is secondary to defects in cellular and ECM components, causing weakness and altered venous tone.[225,255,283] The triggers for these changes remain unclear, although several factors associated with hemodynamic abnormality are likely to be involved, including hypoxia, mechanical stretch and low shear stress.[190,199]

The involvement of MMP and TIMP in vascular diseases is a matter of a strong and continuous scientific interest, especially in the study of effective MMP modulators that would be important in the management of patients with venous diseases.[164] It has been widely documented that the effects of MMP and TIMP on ECM degradation may result in a significant venous tissue remodeling,[225,272] degenerative and structural changes in the vein wall,[115,290] leading to venous dilation and valve dysfunction.[225,254] Taken together, increased MMP activity and altered MMP/TIMP balance[21,224] may also induce early modifications in the endothelium and venous SMC function in the absence of significant ECM degradation or structural changes in the vein wall. In particular, for what concerns CVI it has been suggested that the balance between MMP and TIMP play a crucial role in early steps of varicose vein formation in the lower extremities.[223] In addition, evidence suggests that increased activity

of MMP is also present in the advanced stages of CVI encompassing skin changes and chronic venous ulceration,[215,235,305] as well as in the wound fluid microenvironment.[263,279]

Among proteolysis events, MMPs and their TIMPs have garnered the most attention and have been linked with the pathological events of varicose veins, but different groups have reported contradictory observations.[141,164] Sansilvestri-Morel P. *et al.* 2007[239] showed increased MMP-1, MMP-2, MMP-3, MMP-7, TIMP-1, and TIMP-3, and decreased TIMP-2, in varicose saphenous vein tissues; Ishikawa Y. *et al.* 2000[112] found reduced expression of MMP-1 and MMP-2 in human varicose veins. Different research groups also found discrepant expression patterns of MMP-9, TIMP-1, and TIMP-2.[12,112,139,298] Therefore, although a counter balance between MMPs and TIMPs may affect vein structural integrity.[225] Other families of proteolytic enzymes may also participate in varicose disease development.

Also, Raffetto JD. *et al.* 2008[226] shown than an increase in the level and duration of stretch also evokes adaptation of the SMC phenotype enabling are organization of the ECM, e. g. by altering the expression and activity of MMPs. Consequently, an increase in wall stress stimulates expression of MMP-2 and elevates gelatinase activity both in the media of stretched mouse veins and in human venous SMCs.[77,187] Likewise, MMP-9 activity is increased in varicose veins of human patients[116,] and in rat veins upon exposure to an increased transmural pressure level.[224] This may explain for the fact that the abundance of collagen types I and III is altered in varicose veins as compared to healthy veins.[241,243] In fact, the amount of rigidity-mediating collagen type I is increased in the varicose vessel wall while the distensible collagen type III fiber network is degraded. Recently it is reported that regulatory genes of collagen production are down-regulated in veins affected by superficial reflux disease.[180] In addition, MMPs involved in vascular diseases are the interstitial collagenase, MMP-1, which cleaves fibrillar collagens, which are subsequently degraded by the gelatinases, MMP-2 and MMP-9.[114,201] There are few studies in human tissues which have demonstrated the role of PGE2 on the expression/activation of MMPs.[151,304]

On the other hand, MMP activities are also under control of endogenous tissue inhibitor of metalloproteinase (TIMP) and changes in MMP/TIMP ratio are probably involved

in vascular wall remodeling and in varicose vein formation.[139,141,239] Another group found than active MMP-1 and total MMP-2 concentrations were significantly decreased in varicose veins while the TIMP -1 and -2 tissue inhibitors of metalloproteinases, were significantly increased.[95] In conclusion, such a reorganization of the ECM is a hallmark of the remodeling processes in varicose veins and associated with an increase in rigidity enabling it to with stand a chronic increase in wall stress.[216] A proactive approach to the treatment of the early and late stages of CVI may be focused on the inflammation-related and MMP-dependent proteolysis.[225] In fact, actually the inhibition of MMPs may represent a realistic, novel and possible therapeutic intervention to limit the progression of varicose vein to CVI and leg ulceration.[223] The therapeutic hypothesis is based on the well known role of glycosaminoglycans (specially dermatan sulfate) in health and disease, in wound healing and vein remodeling.[176,275,277]

ECs and SMCs participate in remodeling process of the vessel wall to counter act an increase in wall stress. Dysregulated apoptosis and cell cycle dysfunction occur in varicose veins.[16,70,71,280] The overall number of apoptotic cells and activity are reduced in varicose compared with non-varicose veins.[16,17,40] Dedifferentiation of SMCs maybe due to dysregulated apoptosis.[124]

Besides, abnormalities of the venous endothelium may contribute to venous dilatation and the pathogenesis of varicose veins.[93] Essentially adaptations of the vessel wall, such as an enlarged lumen diameter and remodelling of the ECM, may be evoked by changes in hemodynamic forces such as shear stress and/or (circumferential) wall stress, hence biomechanical stretch. On the other hand, is well stablish that their chronic alteration often promotes cardiovascular pathologies such as hypertension,[195,196] atherosclerosis[138] and venous valve dysfunction.[18]

ECs of varicose veins appear desquamated and degenerated under electron microscopy.[19] Such injured cells are activated and known to release various types of inflammatory mediator and growth.[190,199] Increased expression of inflammatory markers, such as vascular cell adhesion molecule 1, intercellular adhesion molecule 1 and von Wille brand factor, by the endothelium of varicose compared with non-varicose veins has been

recorded.[19,255] In addition, significantly more mast cells, macrophages and monocytes have been observed in varicose compared with non-varicose veins.[125,244,301] Activated leucocytes may also release large amounts of superoxide anions and proteases that are able to degrade the ECM.[190,199] These findings point to the role of ROS in the propagation of varicose disease because their production seems to be further enhanced by local hemodynamic factors.[101]

Another hallmark of both remodeling processes pointing to an involvement of wall stress is the proliferation of medial SMCs. Besides spontaneous responses (contraction or relaxation of SMCs) to temporary changes in blood flow or pressure, a chronic rise in transmural pressure (e. g. during venous hypertension) elicits adaptive vascular remodeling foremost to normalize wall stress. In the comparatively thin-walled and SMC-poor veins, the corresponding structural adaptations of the vessel wall preferentially lead to the typical corkscrew-like morphology of remodeling (varicose) veins that rather points to a (longitudinal) growth between fixed ends.[77]

Endothelial cells (ECs) are directly affected by changes in both hemodynamic forces, whereas only biomechanical stretch stimulates vascular SMCs in the media. Under physiological conditions, both forces stabilize the function of veins and maintain a normotensive blood pressure. In this situation, laminar shear stress-mediated expression and activity of endothelial nitric oxide synthase (eNOS) stimulate the production of nitric oxide (NO). NO as a freely diffusing and membrane-penetrating signaling molecule transmits the rise in laminar shear stress to the SMCs, inhibiting their proliferation and triggering their production of cyclic guanosine 3',5'-monophosphate (cGMP), which subsequently promotes relaxation of these cells and, as a consequence, vasodilatation.[33,198] Furthermore, NO bears several anti-inflammatory effects, as it may react with ROS such as superoxide to form peroxynitrite, thereby inactivating these well-characterized proinflammatory mediators. These findings point to the role of ROS in the propagation of varicose disease because their production seems to be further enhanced by local hemodynamic factors.[101]

INFLAMMATION AND ROS MEDIATORS IN VASCULAR SYSTEM

Growing evidences suggest that an imbalance between pro- and anti-inflammatory mediators is a common pathophysiological mechanism in different cardiovascular diseases.[135,285] Inflammation often begins with endothelial activation through several signal transduction mechanisms, leading to the expression of adhesion molecules which attracts different immune cells.[161] These immune cells, as well as the resident cells, participate as donors and recipients of cytokine signals, amplifying the inflammatory activity.[135,161] This inflammatory environment has deleterious consequences in different functions of the vasculature including contraction/dilation, stiffness and vascular structure.[51,52] We will focus on two pro-inflammatory proteins, and their respective mediators: COX-2 and prostanoids, and NADPH oxidase and ROS.

Biosynthesis of prostanoids begins with the formation of PGG2/PGH2 through the action of COXs on arachidonic acid released by phospholipases from the membrane phosphoglycerides. Different prostaglandin synthases (PGS) metabolize immediately the PGH2 into specific prostanoids. Prostanoids play an important role in inflammation, platelet aggregation, vasoconstriction/relaxation and vascular remodeling. After the initial synthesis of the short-lived but biologically active PGH2, the production of the different prostanoids (the prostaglandins PGE2, PGD2, PGF2α, PGI2 and TXA2) depends on the activity of specific synthases; thus, prostanoid production depends on both COX and PGS activities.

Three different COX isoforms have been described: COX-1, COX-2 and COX-3, where COX-3 is a splice variant of COX-1, thus, COX-3 is sometimes named as COX-1b or COX-1v.[293] In most tissues COX-1 is constitutively expressed under physiological conditions. COX-2 is an inducible isoenzyme and the dominant source of PGs in inflammation. While COX-1 is localized in the endoplasmic reticulum, COX-2 acts predominantly at endoplasmic reticulum and nuclear envelope.[194] In vascular cells, COX-2 expression is induced by a wide variety of stimuli such as IL-1β, ROS, lipopolysaccharide or TNF-α[142,181,269] and it is up-regulated in vascular inflammatory diseases such as aortic aneurysms[127] and balloon-injured arteries.[291,303]

NADPH oxidases (NOX) are the major source of ROS in the vascular wall in both physiological and pathological conditions.[10,68,69,148] NOX are a family of transmembrane oxidases that reduce molecular oxygen to superoxide using energy derived from the oxidation of NADPH/NADH to NADP/NAD. NOX enzyme family consists of five NOX enzymes (NOX1-5) and two Dual oxidases (DUOX1-2). NOX isoforms differ in their inducibility, expression pattern and they also require different sets of accessory proteins for their enzymatic activity.[26]

NOX2 complex is the firstly identified and best studied member of the NOX family. It is expressed by phagocytes (granulocytes, monocytes, dendritic cells) and its expression has also been reported in other cells of the immune system, such as natural killer cells, B cells and mast cells. Lower levels of NOX2 expression has also been detected in T cells.[113,282] Interestingly, recent published data report that neuronal expression of NOX2 regulates stress-response behavior in rats and mice.[245,256]

NOX1 was the first homologue that was described for NOX2.[264] NOX organizer 1 (NOXO1, NCF1 homolog), the organizer subunit of NOX1 and NOX activator 1 (NOXA1, NCF2 homolog) are the cytosolic accessory molecules needed for superoxide production by NOX1 complex.[22,88,268] High expression of NOX1 has been detected on colon epithelium.[266] Additionally, lymphocytes,[266] vascular SMCs,[149] ECs,[133] uterus,[264] placenta,[63] osteoclasts[152] and retina[177] are reported to express NOX1. NOX1 deficient mouse has decreased blood pressure[183] and develop enhanced hyperoxia induced acute lung injury,[45] suggesting an inflammation regulating role for NOX1 derived ROS.

NOX3 is expressed in the inner ear and can produce ROS[53] using both NCF1 and NOXO1 organizer subunits.[23,54] In addition, NOX3 expression has been reported to take place on hepatocytes.[159]

NOX4[53,87,252] was originally identified from kidney, but it is also expressed in many other tissues including ECs[2] and fibroblasts.[57] Interestingly, upon heterologous expression NOX4 is active without cell stimulation and does not require cytosolic subunits for its activity.[182]

NOX5 was identified as calcium activated NADPH oxidase[23] in various fetal tissues, lymphocyte rich areas in spleen and lymph nodes and in uterus and in testis.[24,25,53] The last two enzymes belonging to the NOX family are the thyroid dual oxidase 1 and 2 (DUOX 1 and 2).[64] Contrary to other NOX enzymes and despite of some disagreement in the field, it is likely that dual oxidases convert the produced superoxide directly into hydrogen peroxide.[26] Inactivating mutations in DUOX2 constitute the causative reason for congenital hypothyroidism,[193] highlighting the importance of these enzymes to thyroid function. In addition, there is also evidence that mucosal surface host defense is supported by hydrogen peroxide derived from the DUOX enzymes.[89]

In conclusions, ROS are a heterogeneous group of oxygen radicals and other strongly oxidizing molecules and share common features with closely related reactive nitrogen species. After generation, ROS are further converted into other oxidative species or neutralized by carefully regulated enzymatic and non-enzymatic antioxidative reactions. Free radicals such as ROS are atoms or groups of atoms with an unpaired number of electrons. Examples of free radicals are hydrogen peroxide, hydroxyl radical, nitric oxide, peroxynitrite, singlet oxygen, superoxide anion and peroxyl radical. Free radical formation is increased by immune cell activation, inflammation, ischemia, infection, cancer, and chronic heart disease. The radicals react with various cellular components including DNA, proteins, and lipid/fatty acids which leads to DNA damage, mitochondrial malfunction, cell membrane damage and eventually cell death (apoptosis). Molecular oxygen (O_2) is converted into superoxide anion (O_2^-) by the action of specialized enzymes, in mitochondria during cellular respiration, by ionizing and UV radiation and during the metabolism of a wide range of xenobiotic substances and drugs.[296] Superoxide can either spontaneously or by the action of one of the three superoxide dismutases (SOD1-3) be further converted into hydrogen peroxide (H_2O_2). The reaction between superoxide and nitric oxide produces peroxynitrite (ONOO−), converging reactive nitrogen and oxygen species metabolism.

In relation to inflammatory mechanisms and ROS production in varicose veins, stretch-stimulated ECs or SMCs increase the expression and activity of certain NADPH oxidases and thereby production of ROS, which in low to intermediate amounts trigger signalling

pathways sensitive to oxidative modification of pivotal effector proteins.[18] This is quite in analogy to processes elicited by arterial hypertension, where ROS are a major component in the initiation of SMC dedifferentiation,[214] partially by the increase in H_2O_2 formation,[262] and additionally support a pro-migratory and growth promoting phenotype.[42,153]

Interestingly, activation of the transcription factor activator protein 1 (AP-1) appeared to be a prerequisite for venous remodeling, proliferation and MMP-2 expression in this context, as blockade of its activity essentially abolished all these processes. The relevance of wall stress for venous remodeling is further underlined by the fact that AP-1 is activated by ROS, namely hydrogen peroxide (H_2O_2), whose intracellular concentration is controlled by biomechanical stretch.[216] Stretch-stimulated ECs or SMCs increase the expression and activity of certain NADPH oxidases and thereby production of ROS, which in low to intermediate amounts trigger signaling pathways sensitive to oxidative modification of pivotal effector proteins.[18,30]

During varicose vein development, the release of NO would inhibit the pro-inflammatory activation and proliferation of ECs and SMCs, which is promoted by an increase in wall stress. Moreover, NO would neutralize ROS such as superoxide anions, whose production is elevated by a wall stress-dependent stimulation of NADPH oxidase expression and activity. In addition, prevention of superoxide dismutation to H_2O_2 would simultaneously limit the activity of AP-1, as has been shown to be the case in arterial hypertension.[216,271] In fact, enhanced ROS production has recently been verified in varicose veins,[140] suggesting that this mechanisms may play a crucial role in the pathogenesis of the disease.

Inflammatory cell infiltration into the vascular wall is an important feature of varicose veins,[300] and its central roles are broadly evident in vascular diseases,[32,162,257] that was principally demonstrated in arterial pathology, characterized by the presence of macrophages,[166] T cells,[132] and mast cells[265] in the intimal arteriosclerotic lesions. In the adventitia of varicose veins Xu N. *et al.* 2014[300] demonstrates increased tryptase-positive mast cells infiltration. Several studies have demonstrated that mast cells and their specific proteases, tryptases and chymases, activate MMPs;[234] convert angiotensin-I to angiotensin-

II;[163] degrade ECM fibronectin;[273] and induce SMC detachment and apoptosis.[273] Also, Xu J & Shi GP found that T-cell numbers significantly increased in varicose veins compared with non-varicose veins,[299] although Sayer GL & Smith PD showed no differences at T-cells number in varicose veins.[244] But, when they studied macrophages CD68+, they didn't find significant differences in macrophages at varicose veins. On contrary, Nicolaides AN found infiltration of valve leaflets and the venous wall by leukocytes (monocytes and tissue macrophages) and also an increased number of mast cells and neutrophils).[203] Over expression of inducible nitric oxide synthase and transforming growth factor-β1, as well as increased presence of CD68+ monocyte/macrophages, has also been documented in patients with varicose veins.[117]

In summary, this increased number of inflammatory cells that both inflammation and degradation of ECM proteins would be crucial in the early etiopathogenesis of CVI.[267] Independently of the cellular type involved, a generally accepted theory is that inflammatory process and weakness or alteration of the matrix components of the vein wall play an important role in the pathogenesis of CVI.[171,204,242] The primary CVI is an inflammatory pathology that involves an erratic structural remodeling in the venous well leading to vascular incompetence and the development of varicose vein, characterized by altered collagen and elastin content. In the early steps of varicose vein formation is crucial the role of MMP/TIMP balance, due to the increased secretion of proteolytic enzymes, implicated in both ECM and vascular degradation during inflammation processes, from vascular cells and inflammatory cells (like macrophages, neutrophils and mast cells).[115,118,203] The inflammatory mechanisms are also corroborated by animal models in which acute and high intravenous pressure levels appear to promote pro-inflammatory responses,[102,103,146] suggesting that they may be a consequence rather than the cause of advanced varicose vein development. However, new research are necessary in relation to the participation of inflammatory mechanisms in the pathophysiology of varicose vein, because in a recent publication, Gomez I. *et al.* 2013[94] found the absence of COX-2 in the varicose veins.

Prostanoids, including prostaglandins (PG) and thromboxane, has been rarely investigated in the context of varicose veins. Prostanoids are produced by most blood and

vascular cell types.[79] PGE2 via selective activation of EP1-4 receptor subtypes is involved in the control of vascular tone,[80] inflammation,[43,96] pain[260] and vascular wall remodeling.[151] PGE2 plays a major role in vascular wall remodeling and in collagen overexpression.[95] PGE2 is synthesized from arachidonic acid (AA) through the enzymatic activities of two cyclooxygenases (COX-1 and/or COX-2) and three PGE synthases (PGES).[212] Among the three PGES that specifically catalyze the final step of PGE2 biosynthesis; two are constitutive: microsomal (mPGES-2) and cytosolic (cPGES).[43,99] The third, mPGES-1,[43,99] is quantitatively the most important enzymatic activity for PGE2 production. mPGES-1 and COX-2 expression are generally co-induced by inflammatory cytokines such as IL-1b.[119]

In varicose veins, this reduced PGE2 content and the lower density of its receptor (EP4) are responsible for the down-regulation of the MMP/TIMP ratio.[95] The consequence of this biological cascade is a reduction of active collagenase content and an accumulation of collagen in the vascular wall of varicose veins and could explain the intima hyperplasia and the thickening observed in the varicose wall.[20,205] This phenomenon is in complete accordance with a recent publication where selective deletion of mPGES-1 in both EC and vascular SMC resulted in hyperplasia by enhancing the neointimal proliferative response to vascular injury in mice.[52] Also, Gomez I. *et al.* 2014[95] showed that cPGES protein was not found in all sample and was only detectable at the mRNA level. In contrast, mPGES-2 protein was found at similar levels in all venous preparations. These results[95] concerning mPGES-1 were unexpected since they found this enzyme in the SV while mPGES-1 expression is classically induced by inflammatory conditions via NF-kB.[96] In conclusion, the reduction of PGE2 concentrations in human varicose veins is due to a decrease in mPGES-1 and an increase in 15-PGDH. These effects lead to the imbalance of vascular wall remodeling by decreasing the MMP/TIMP ratio and could result in the accumulation of collagen in varicose veins. This endogenous mechanism could be a protective effect of the saphenous vein in order to restrain the blood stasis by reinforcing the vascular wall, avoiding ectasic segment formation and venous wall rupture.[95]

The aim of the present Doctoral Thesis is the study of histological, immunohistochemical, ultrastructural and morphometrical alterations that success at

varicose veins and tries to connect with the primary events that triggers with the evolution of the disease. In the present Thesis we propose a new procedure to evaluate of confocal microscope structure of the endovascular surface and intimal surface of *in toto* fixed surgical specimens of saphenous vein with varicose lesions. This method can be demonstrated the panoramic reconstruction of different grades of intimal varicose lesions. In our knowledge, this procedure has not been previously apply to normal and pathological vein research. Nowadays, as we have shown, literature presents controversy about the role of inflammation, ROS or the mechanism of genes involve in ECM production. We would like to clarify, or at least, support with arguments the implication or absent of this hypothesis in the evolution of varicose veins. For that intention, we have used histological, biological and molecular methods in our study for a global vision of the varicose disease.

HIPHOTESIS AND OBJECTIVES

HYPHOTESIS

¿Are the histological and molecular changes of varicose vein regulated by molecules involved at oxidative stress pathways?

OBJECTIVES

1st. To describe histological and immunohistochemical distribution of elastic fibers, smooth muscle actin, collagen type I and collagen type III at the proximal and distal segment of varicose saphenous veins with intimal lesions.

2nd. To analyze the evolution of the proportion of the different proteins; elastic fibers, smooth muscle actin, collagen type I and collagen type III, in relation to fibrotic mechanisms develop in the intima of varicose vein.

3rd. To study the confocal microcopy reconstruction of the intimal distribution of elastic fibers and collagen in varicose veins, and their possible correlation to intimal fibrosis demonstrated by ultrastructural study.

4rd. To evaluate the molecular mechanism of oxidative stress by the H_2O_2 production, NADPH activity and the mRNA expression of NOX-1, NOX-4, COX2, mPGES, MAC3, collagen type I, collagen type III, elastin and SMA.

MATERIAL AND METHODS

MATERIAL

The aim of the study was evaluated immunochemistry and molecular expression of molecules related to extracellular matrix, ROS stress and inflammation in human varicose veins. 20 saphenous varicose veins removed by phlebectomy from patients with chronic venous insufficiency (CVI) and 10 saphenous normal veins from patients who suffer a limb amputate were studied. Veins were divided in two groups: proximal segment and distal segment (Figure 3). The proximal segments were extracted near the saphenofemoral junction, and the distal segments near the malleolus area. Subjects were matched for age and sex, known from previous studies to affect vascular oxidative stress and superoxide production.

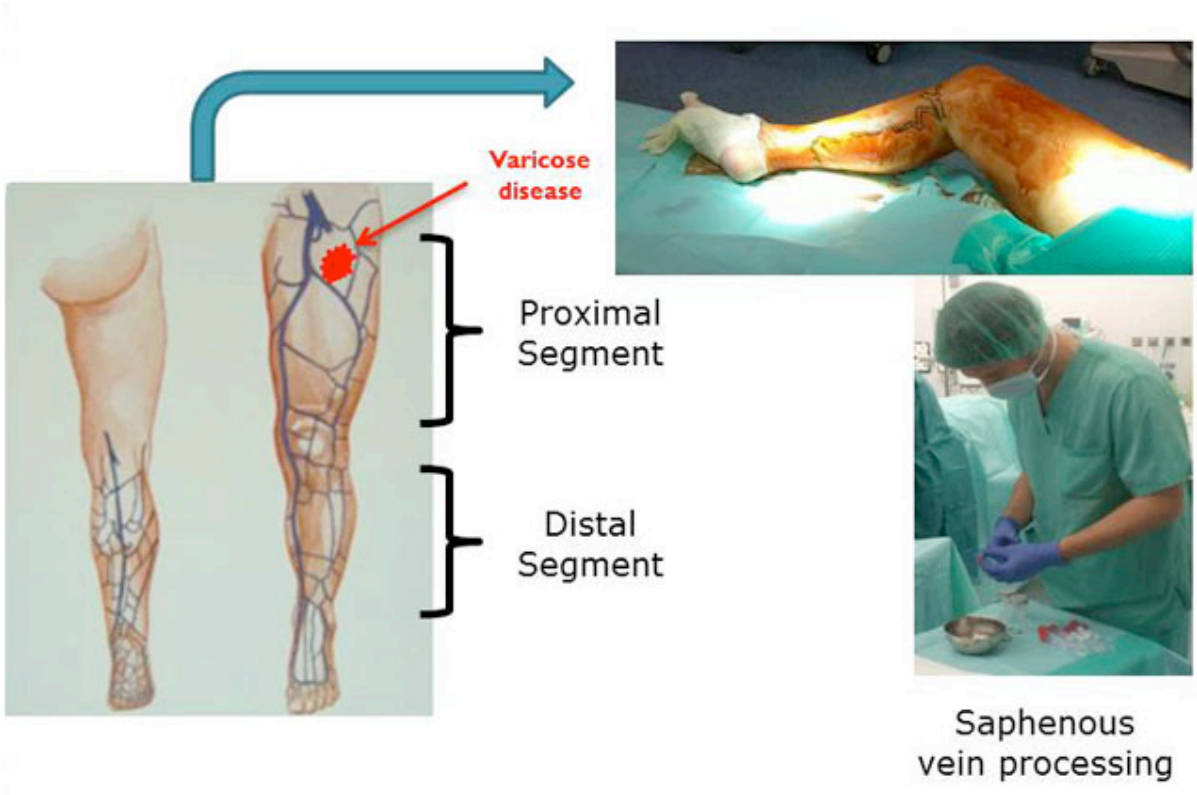

Figure 3. Histological and molecular processing of both proximal and distal saphenous vein segments realized by Juan Velasco into surgical room.

METHODS

Ethical aspects

Use of human varicose veins and normal veins samples, had been approved by the Ethics Committee of Clinical Investigations of the La Paz Hospital and Moncloa Clinic, with full informed consent from the patients.

Tissue processing

Veins full removed were sectioned perpendicular to main axis for extracted three pieces of approximately 5 mm each from proximal and distal segment. Pieces were dehydrated by immersion at increasing concentrated alcohols (70%, 96% and 99%) and after that into butyl acetate two times per one hour each. Finally, tissues were embedded in paraffin wax for two hours.

Histological methods

Veins were fixed in 4% buffered formalin during 48 hours and embedded in paraffin wax. 5 slices from deparaffinized tissue sections were stained with hematoxylin-eosin, Masson´s trichrome and orcein (for elastic fibers detection), using standard procedures (Figure 4). As mounting methods, the water-free mounting medium DPX (Probus, Badalona) was used.

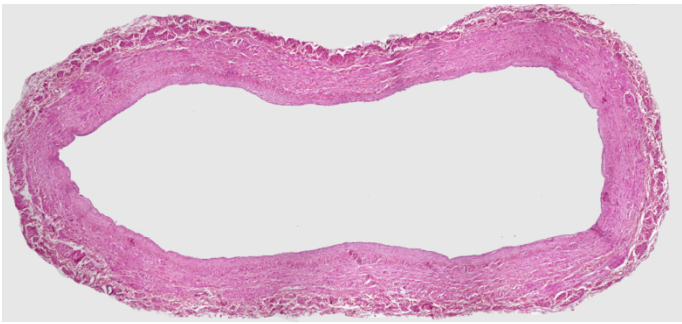

Figure 4. Panoramic view of a transversal section of a varicose saphenous vein with thickened of the intima layer and moderate fibrosis of the media layer. Hematoxylin-eosin stain

Immunohistochemistry (Streptavidin Biotin Peroxidase Method)

Human veins were fixed in 4% formalin for 48 h, embedded in paraffin and prepared in 5 µm cross sections with a microtome. Sections from saphenous veins were de deparaffinized in xylene and rehydrated in graded ethanol solutions. Slides were then rinsed in distilled water and treated with 3% hydrogen peroxide in distilled water for 10 min to remove endogenous peroxidase activity. Slices were washed at PBS at room temperature for 5 minutes. Then, slices were immerse at citrate buffer (pH 7,6) and introduced two times for 2.5 minutes into a microwave for epitope detection. After that, when slices were again at room temperature, they were washed with distilled water. Sections were blocked with a 10% of normal serum in PBS for 20 min and incubated with anti-SMA (1/400), anti-collagen-I (1/400), anti-collagen-III (1/400), anti-collagen-IV (1/400) and anti-vimentin antibodies overnight at 4°C at a humidity chamber (Table 2). Antibodies were diluted at PBS+BSA solution (1%). After washing 3 times in PBS samples were incubated for 1 h with a biotinylated secondary antibody. After rinsing 3 times in PBS, streptavidin-biotin-peroxidase complex was applied, and the slides were incubated for 30 min. Color was developed using 3,3'-diaminobenzidine and sections were counterstained with hematoxylin before dehydration, clearing, and mounting with DPX (Figure 5). Negative controls in which the primary antibody was omitted were included to test for non-specific binding.

Antibody	Company	Dilution
SMA (monoclonal mouse anti-smooth muscle actin)	DAKO	1:400
Collagen I (monoclonal mouse anti-human Collagen I)	ABCAM	1:400
Collagen III (monoclonal mouse anti-human Collagen I)	ABCAM	1:400
Vimentin (monoclonal mouse anti-human Vimentin)	ABCAM	1:200

Table 2. Antibodies used.

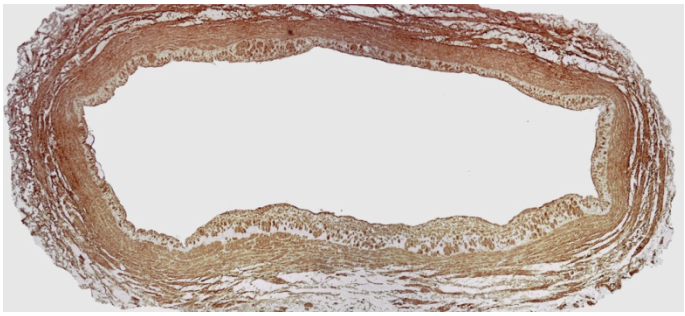

Figure 5. Alpha Smooth Muscle Actin, SMA, expression in a saphenous vein with intense varicose lesions.

Electron microscopy methods

For electron microscopy study, the specimens were cut into small blocks (1 mm^3), fixed in Karnovsky´s fixative, post-fixed in 1% phosphate buffered osmium tetroxide for 2 hours, dehydrated in ethanol and embedded in Epon-812. Sections 1 µm thick are stained with toluidine blue. Some ultrathin sections were stained with uranyl acetate and lead citrate and then studied in a Philips 300 electron microscope (Figure 6).

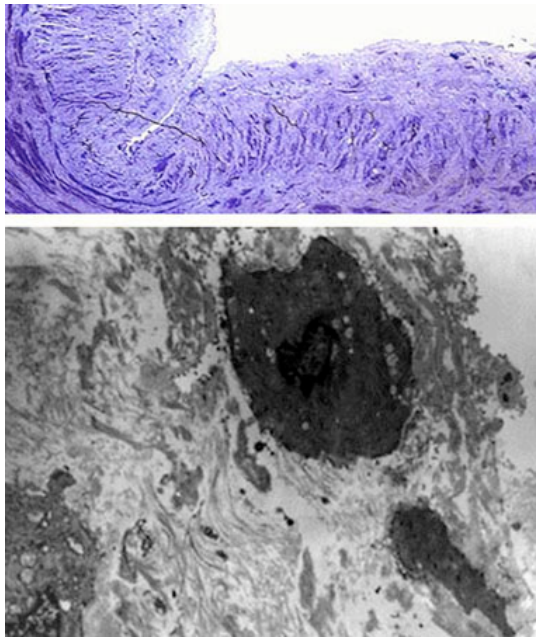

Figure 6. Ultramicrotome section of a varicose saphenous vein stained with toluidina blue, and electron microcopy photography of thickened intima in a varicose vein

Fluorescence methods for elastic fibers

Human veins were fixed in 4% formalin for 48 h, embedded in paraffin and prepared in 5 µm cross sections with a microtome. Sections from aorta were deparaffinized in xylene and rehydrated in graded ethanol solutions.

Elastic fibers can be studied by their autofluorescence activity. We evaluated elastic fiber organization in 5 µm cross sections. We used a fluorescence microscopy Leica DMLB and installed with a fluorescence light power ebq 100. Vein segments were watched at λ 482/560 nm and photographed with a 40x objective. Images were taken with a Leica DC 200 digital camera. Photographs of the internal elastic lamina and elastic fibers were taken randomly for a better study of the tridimensional organization (Figure 7).

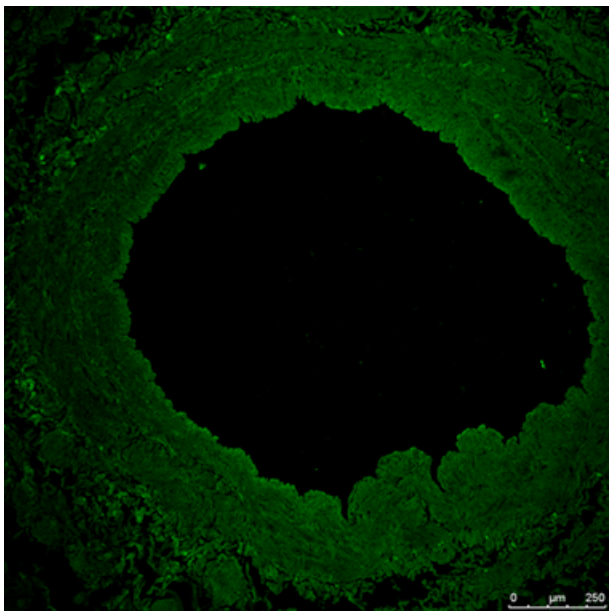

Figure 7. Autofluorescence of a normal distal segment of saphenous vein

Confocal microcopy methods for in toto study of elastic fibers and collagen tissue, using an endovascular vein wall reconstruction

In the present study we realized a new procedure to evaluate the structure of the endovascular surface of saphenous vein with varicose lesions examined in confocal microscope. In our knowledge, this procedure not previously is applicate to normal and pathological vein research. Moreover, we design an easy to implement and relative fast

methods for valuation of intimal surface in normal endovascular areas and in others areas that present intimal thickening secondary to chronic varicose disease. Conventional stereoscopic microcopy permit visualized the endovascular surface of vein using surgical specimens open longitudinally, and permits distinguish the normal intima to the fibrotic intimal areas. However the morphological information obtained with this classic microcopy method is poor. In the present Thesis, the use of confocal microcopy demonstrated the different grades of intimal varicose lesions (Figure 8a). In the fibrotic areas associated to intimal varicose lesions, we merge confocal image from Z axis at the superior level (80-90 µm deep) from the intimal thickening. In this way we evaluated the histological correlation of elastic autofluorescence, with light ray lase reflection for collagen detection and nuclear DAPI stain for distribution of nuclei into the vein intima. In a determinate confocal Z axis section, we merge image from elastic autofluorescence, light ray reflection and DAPI. Image can be rotated to a plan perpendicular to the luminal surface. This image also demonstrated the histological structure from two randomly perpendicular plans respect the intimal surface view (Figure 8b). In conclusion, the autofluorescence proprieties of elastic fiber observed in Leica SP5 Laser Microscopy, using a laser ray at 488 λ, combined with the application up the same vein specimen of reflexed laser ray, for view the distribution of collagen fibers into the connective tissue present in the lamina propria of the intima determinate a new diagnostic methods for intimal fibrosis evaluation *in toto* of surgical specimens of veins with intimal lesions (Figure 8b).

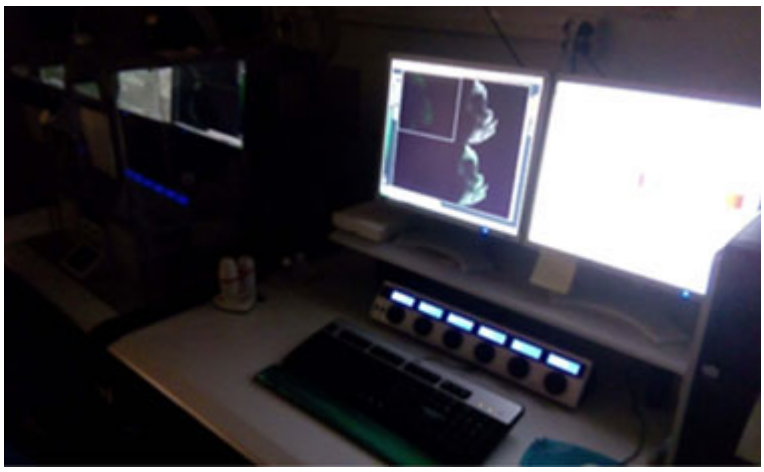

Figure 8a. Confocal microscopy study of endovascular surface of *in toto* specimen of a saphenous vein segment. In the screen, view the image confocal capture.

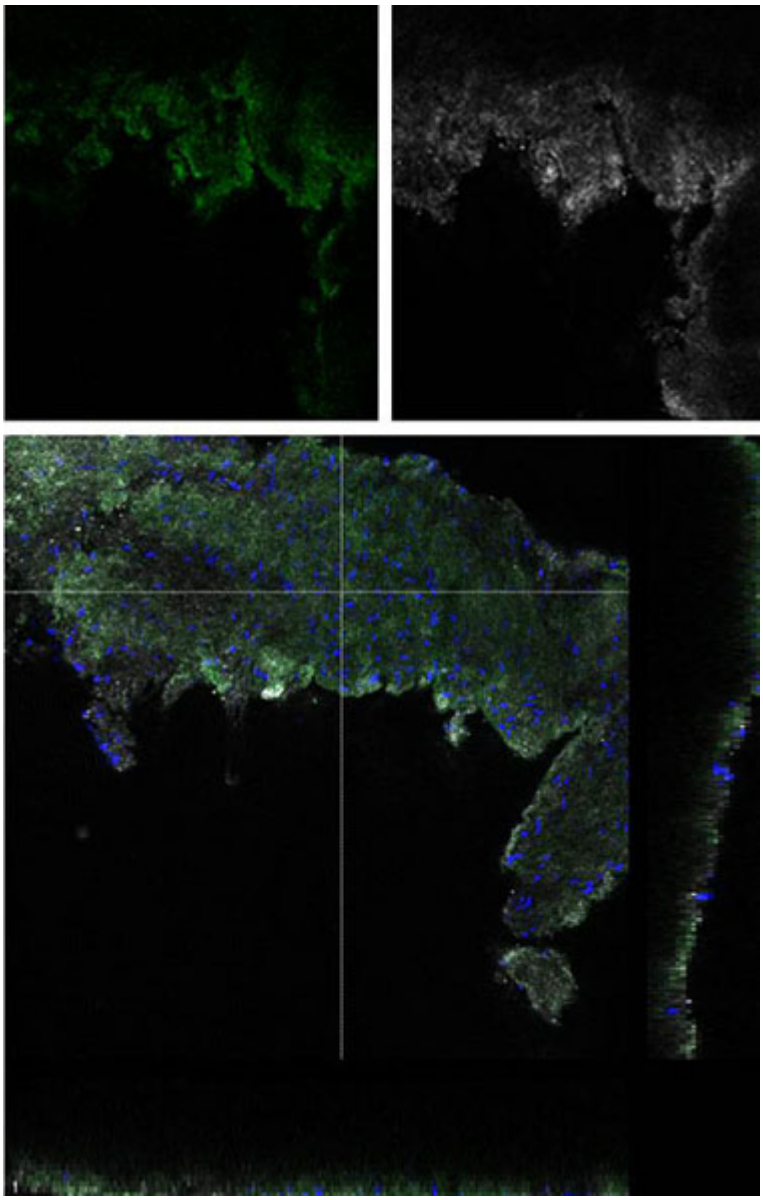

Figure 8b. Confocal microscopy study of endovascular surface of *in toto* specimen of a saphenous vein segment. The two superior images respectively correspond to disposition into the intima of elastic amorphous material (green color) and collagen distribution (grey color). The inferior image correspond Merge view of nucleus (blues) elastic material and collagen and a reconstruction of the same specimen.

Histological quantification

Vein slices were previously examined by two pathologists for select slices without artifacts. Afterwards, vein wall areas at each slice were randomly selected. 10 microscopic fields per vein wall area were delimited.

For fields selection, we used a method called “randomly selection”, that it is a non-biased systematic random sampling method. A grid divided at same 100 areas was used. Each gap had assigned a number between 00 and 99 and then we used a random number table for select 10 numbers. Those methods guarantee a non-bias selection. Each field selected was photographed with the 40x objective. At every field selected, intima layer were morphometrically studied from slices immunochemistry labelled with anti-SMA antibody, anti-collagen-I antibody, anti-collagen-III antibody, and anti-collagen-IV antibody and also for the elastic fibers study, orcein stain or autofluorescence activity were used.

All images were acquired at room temperature using a microscope (Leica Eclipse 55i) mounted with a digital camera (Leica DC200) and photographs were acquired by Image-Pro Plus software at TIFF format. All images were done with a 40x objective. At all selected fields were photographed intima and media tunica. Afterwards, images were processed with ImageJ (http://rsb.info.nih.gov/ij) software. ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health. We used ImageJ for quantify expression of different proteins at intima layer.

ImageJ was designed with an open architecture that provides extensibility via Java plugins and recordable macros. ImageJ had been previously used by other investigators for histological quantifications. Nowadays, ImageJ plugins make it possible to solve many image processing and analysis problems, from three-dimensional live-cell imaging to radiological image processing, multiple imaging system data comparisons to automated hematology systems. ImageJ's plugin architecture and built-in development environment has made it a popular platform for teaching image processing. Nowadays, thousands of new plugins are develop for users for solve new problems. Also, ImageJ can display, edit, analyze, process, save, and print 8-bit color and grayscale, 16-bit integer, and 32-bit floating point images. It can read many image file formats, including TIFF, PNG, GIF, JPEG, BMP, DICOM, and FITS, as

well as raw formats. ImageJ supports image stacks, a series of images that share a single window, and it is multithreaded, so time-consuming operations can be performed in parallel on multi-CPU hardware. ImageJ can be used at Windows, OS X or Linux operating systems.

To a correct morphometric study of area occupied by SMA, collagen-I, collagen-III, collagen-IV and elastic fibers compared to total intimal area at varicose and normal veins at both segments we used the ImageJ plugin color deconvolution, develop by Gabriel Landini (Landini G: Colour deconvolution plugin 1.5. http://www.dentistry.bham.ac.uk/ landinig/software/ cdeconv/cdeconv.html). Histological stains are “light absorbing dyes” so can be considered as being subtractive color. The plugin requires images to have a neutral background to work properly. Vectors should be worked out from single-stained control slides. So, we could separate original images into three different channels, in our case Hematoxylin channel, DAB channel and a complementary one. We studied DAB channel (8 bits image) for quantification of immunochemical positive area of SMA, collagen-I, collagen-III, collagen-IV and for elastic fibers study we used. Then, we split up image into intima layer and rest of vascular wall, so we studied only intima layer. After plied color deconvolution and select intima layer, we decided a threshold between 0 and 255 (because 8 bits images works between 0 (minimum light, black) and 255 (maxima light, white)), and we used same threshold at every marker. Subsequently, we quantified immunochemistry positive area for each protein, SMA, collagen-I, collagen-III, collagen-IV at intimal layer. We did also the same for positive stain for elastic fibers. We measure as well total intimal area at each photography. Results were expressed as proportion between positive stain area and intimal total area (%).

Molecular methods

RNA Analysis

For varicose veins mRNA extraction, veins were homogenized on ice in 1 mL of Tri Reagent using a Polytron PT-20 (Kinematica AG, Lucerna, Switzerland). Samples were centrifuged 5 min 12,000 g to remove the debris of tissue, supernatants were transferred to a fresh tube and total RNA was obtained according to the manufacturer’s recommendations.

Total RNA absorbance was determined using a spectrophotometer Nanodrop (Thermo Fisher Scientific Inc, Wilmington, DE, USA) at 260 nm (1 unit A260 de ssRNA = 40 µg/mL). Sample absorbances at 280 and 230 nm were also determined in order to check RNA quality. 1 µg of total RNA was reverse transcribed using High Capacity cDNA Archive Kit (Life Technologies Inc., Gaithersburg, MD, USA) with random hexamers in a final volume of 10 µL according to manufacturer's recommendations. The reverse transcription PCR protocol was 10 min at 25 ºC, 2 h at 37 ºC and 5 min at 85 ºC.

Quantitative PCR (qPCR) was performed in a 7500 Fast ABI System (Life Technologies). Depending on the gene, primers for SyBR Green or TaqMan Probes were used. The final volume of the reaction was 10 µL composed by 5 µL of sample (50 ng), 4 µL of iTaq FAST SyBRGreen Supermix or 4.5 µL of iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA, USA) and 1 µL of forward and reverse primers or 0.5 µL of each probe. SyBRGreen primers concentrations were tested with a standard curve to obtain efficiency between 90-110%. We used Taqman Gene Expression Assays for human COX-2 (Hs00153133_m1), mPGES-1 (Hs00610420_m1), NOX-1 (Hs00246589_m1), NOX-4 (Hs00418356_m1), TXAS (Hs01022706_m1). SYBR green primers sequence information is listed on Table 3.

Genes	Sense (5'-3')	Antisense (5'-3')	Concentration (nmol/L)
SMA	TTCGTTACTACTGCTGAGCGTGAGA	AAGGATGGCTGGAACAGGGTC	1:500
Collagen I	CAGCCGCTTCACCTACAG	AATCACTGTCTTGCCCCAGG	1:500
Collagen III	CCAGGAGCTAACGGTCTCAG	CAGGGTTTCCATCTCTTCCA	1:500
Elastin	GGAGGACTCGGAGTCGGAG	CCAGCAGCACCGTATTTAGCT	1:700
MAC 3	GGTTAATGGCTCCGTTTTCA	TCATCCAGCGAACACTCTTG	1:500
COX 2	GCTCAGCCATACAGCAAATCC	CCAAAATCCCTTGAAGTGGG	1:500
β actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG	1:500

Table 3. SyBR Green primer sequences with the name of the target gene, the specie and the final concentration. β-actin.

PCR cycles proceeded as follows: initial denaturation for 30 s at 95°C followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Melting curve analysis was performed in SYBR green reactions to show PCR product specificity. Relative expression was determined using 2-ΔΔCt method (where Ct is threshold cycle),[168] using β-actin rRNA as the internal control. Variations of mRNA levels were calculated as fold increase over controls or over controls with the corresponding inhibitor.

NADPH oxidase activity

The $O2^{\bullet-}$ production generated by NADPH oxidase activity was determined by a chemiluminescence assay using lucigenin. Lucigenin is an aromatic compound, which can be reduced by $O2^{\bullet-}$ producing light. This light is read by a luminometer.

H_2O_2 release

The Amplex Red reagent in combination with horseradish peroxidase (HRP), has been used to detect H_2O_2 released from biological samples. In the presence of peroxidase, the Amplex Red reagent reacts with H2O2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Resorufin has excitation and emission maxima of approximately 571 nm and 585 nm respectively and the signal can be measured fluorometrically or spectrophotometrically.

Tissues were homogenates in phenol red-free medium. In order to prevent interference with the resorufin measurement, we used phenol red-free medium. The homogenate were used to determine H_2O_2 release and to measure total protein content. Amplex Red (100 µmol/L; Sigma-Aldrich) and horseradish peroxidase type II (0.2 U/mL; Sigma-Aldrich) were added to 50 µL of supernatants. Fluorescence readings were made in duplicate in a 96-well plate at Ex/Em = 530/580 nm. H2O2 concentration was estimated using a standard curve between 0-4.8 µmol/L of H2O2. Total protein of cell lysates as well as the volume of the supernatants was measured in order to normalize H_2O_2 values.

Microscopy photography

Microscopy photographs were done by a digital camera Leica DC200, saved as TIFF and process with Adobe Photoshop CS4. Photograph pieces were done with program Quark X Press Passport 6.0 software.

Data analysis and statistics

Data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All values are expressed as mean ± SEM of the samples used in each experiment. Statistical analysis was done by Student's *t* test, Mann-Whitney test or by one-way ANOVA followed by a Bonferroni test. Values were considered to be significant when $P<0.05$.

RESULTS

All varicose veins samples studied were from patients at 2-3 CEAP stage, whose went to surgical procedure. For histological study, we divided the vein in a proximal segment (saphenous-femoral junction) and in a distal segment (near ankle). Each segment was preferentially evaluated by transversal sections, but also few of them were evaluated by longitudinal sections, for a better mapping of the intimal varicose alterations. Obviously, due to the varicose lesion is multiple along the length of the vein, areas without lesion or with a minimal lesion were also evaluated.

Those cases with a well-established varicose lesion always have a moderate or intense intimal thickening. These areas are associated to an increase of connective tissue in the media layer and to multiple areas of muscular atrophy (Figure 9). Deposits at the intima of dense irregular connective tissue surround the SMCs and nests of spindle or circle cells are located in the superficial region of the intima. The ECs from these veins are normal, although in multiple histological sections the endothelial epithelium has disappeared. (Figure 10).

Our immunohistochemical study shows two cell populations at the intima. The basal region of the intima has a strong stain of SMA at the cytoplasm of the transverse cut SMCs. Likewise the undifferentiated cells from the top region has a strong stain for SMA at their cytoplasm, although most of them are small cells and present a thin line around their cytoplasm (Figure 11). In addition, the immunoexpresion of SMA is strong at the leiomyocytes in the medial layer that allows us to see the limit of the SMCs and to differentiate them from the interstice of the connective tissue (Figure 10). The use of a monoclonal antibody against vimentin allow us to identify the presence of intermediate vimentin filaments stain the scant cytoplasm of the spindle or rounded cells present at the surface of the intimal layer (Figure 12). The use of seriated slices allows observing undifferentiated cells nests that co-expressed both vimentin and SMA molecules. It is important to point out the high number of vimentin + cells at the intima and the limited number of vimentin + fibroblast organized at the interstice of the connective tissue at the media layer (Figure 12).

To identify the histological type of the proliferative cells from the intima we studied the ultrastructure from the proximal and distal segments of the varicose veins. The study of

the semithin cuts confirm the wide deposition of ECM in all cases with intimal thickening and the presence of undifferentiated cells and nests of spindle and round cells, most of them localized at the third top of the intima. The ultrastructure of few of these cells corresponds to fibroblast, but most of them are myofibroblast, due to their cytoplasm is shorter than the fibroblast cytoplasm. Also, at the periphery, we observe focal areas with higher electronic density associated to the cytoplasmic membrane that can be interpret as anchor electrodense deposits near to the cytoplasmic membrane, characteristic feature of leiomyocytes and not observed in fibroblast (Figure 13 y 14).

The use of confocal microscopy, at histological section and also from the endovascular surface to connective tissue under the endothelium (*in toto* vein samples), allow us to organize the spatial disposition of the fibers and the ECM at different sections of the intimal lesion. The laser emission (λ=488nm) over the intimal wall from veins *in toto* and in microtome sections permit to identify the autofluorescence signal from elastic fibers. At all our material we have confirmed that the elastic fibers are scant at the thickening intima but with signals of amorphous elastic material (Figure 15). These materials are immersed at the ECM with the presence of thin collagen fibers visualized by the refraction of the laser emission (Figure 15 and 16). The study of different level from the confocal microscopy permit to confirm the relation between the elastic material with the ECM and the existence of thin and short collagen fibers whose are widely distributed for all the intimal area (Figure 17).

The expression of collagen type III is different between the proximal and the distal segment in the intima from varicose veins. The proximal segment has a higher intimal thickening than the distal segment with a strong stain for collagen type III. Also, at the medial layer exists a weak stain for collagen III, where the collagen fibers surround the SMCs (Figure 18). The distal segment has also shown stain for collagen type III, although the intimal thickening is smaller than in the proximal segment. The medial layer has a weak stain for collagen III that it is deposited in the connective tissue near the SMCs (Figure 19). Additionally, they are differences at collagen type I expression between the proximal and the distal segment. The expression of collagen type I is more intense at the intimal level than at

the medial level (Figure 20). It is necessary to point out that the immunoexpresion intensity of collagen type III and collagen type I are higher at the subendotelial level than in deeper areas of the intima. In summary, this strong signal of collagen determinate an intense fibrosis at the intima subendothelial surface (Figure 18-20).

At the areas with thickening of the intimal venous layer we have demonstrated the presence of elastic fibers by two methods: by orcein stain and by autofluorescence at confocal microscopy. Both methods allow us to identify an internal elastic lamina, but at multiple histologic areas this internal elastic lamina is lost and it is substituted by fibrous tissue. Only at deeper areas of the intimal thickening we can observe tortuous and long elastic laminas, while at the medial and superficial third the material elastic is deposited as an amorphous and granular material related to limited, thin and short elastic fibers (Figures 21 and 22).

Fig. 9. Saphenous varicose vein with different fibrosis grades at the wall

a: Histological image from a transverse section of the proximal level in saphenous vein. We can show a big thickening of the intima. H-E.

b: At the same segment can be seen a rise of the connective tissue that cause multiple fibroses nodules at the intimal wall. Also, the medial wall presents an irregular thickness, with deposit of fibrotic tissue and a decrease of the muscular wall. Masson's trichrome.

c: Detail from the image before. We can see the different grade of intimal fibrosis and the atrophy from de SMCs at the medial layer. Masson's trichrome.

d & e: The fibrosis nodules from both sides of the vein causes an evident narrowing of the vascular lumen. This fibrous tissue is mainly form by dense irregular connective tissue with domination of fibers and ECM. Also, the cellular density at the intimal layer is big. Masson's trichrome.

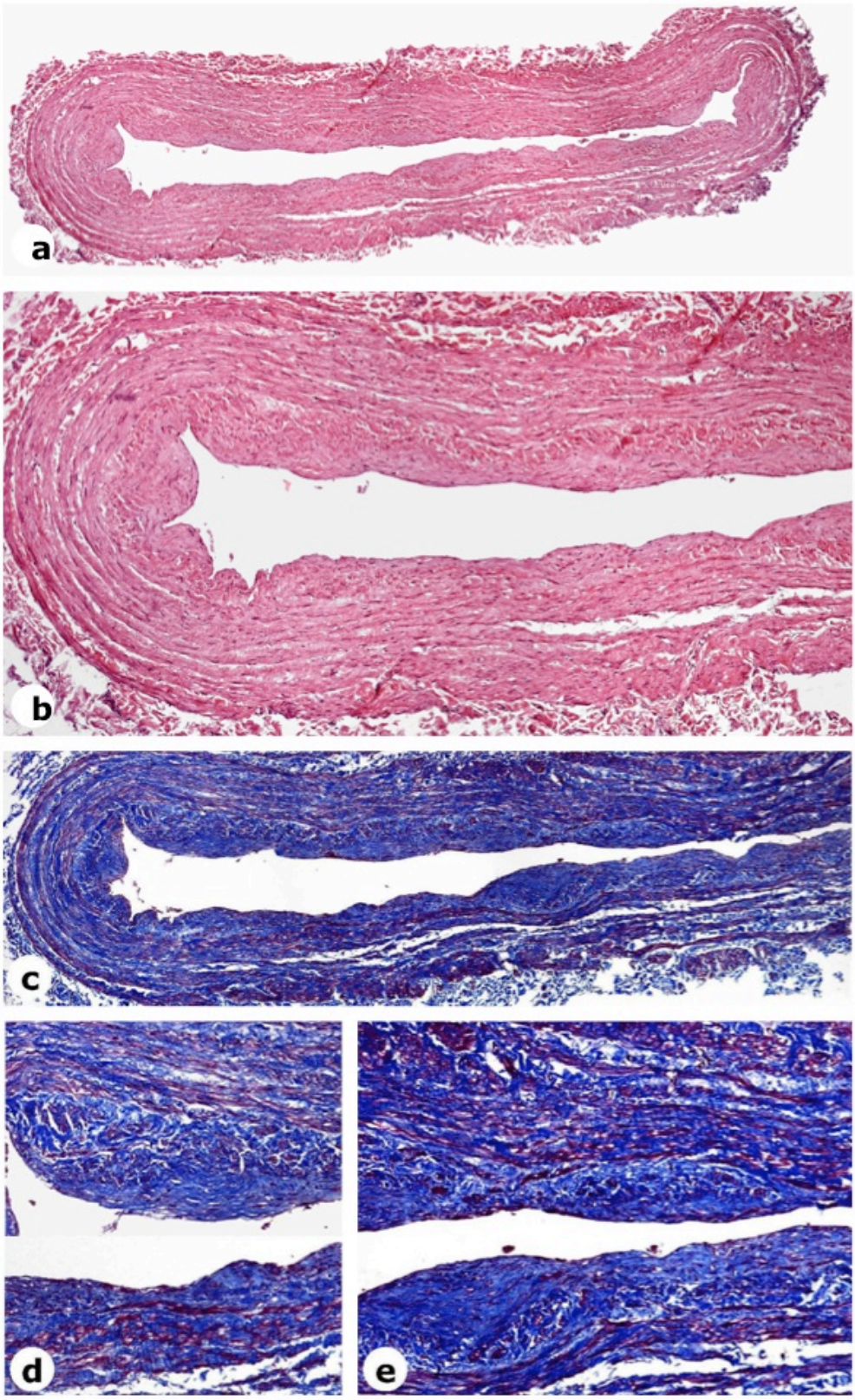
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Fig. 10. Fibrosis and cellular proliferation at the intimal layer, associated to muscular atrophy at the medial layer in a saphenous vein with moderate varicose lesions

a: Longitudinal section from a saphenous vein. It observes a moderate rise of the intimal thickening. The endothelial layer has disappeared. Also, it is seem a deposition of dense irregular connective tissue and rise of the intimal cells at the intimal layer. Masson's trichrome.

b: Detail from the image before. We can identify few oval or spindle cells with limited fibrillary cytoplasm at the intimal layer. These cells are immersed at a dense connective tissue where we can see fibrillary components and abundant ECM. At the medial layer, the fascicules of the SMCs are very irregular and the leiomyocytes presents a small diameter. The muscular fascicules from the medial layer are also circle by connective tissue. Masson's trichrome.

c: Parallel section from the image before. The thickness of the intimal is moderated and it is form by ECM and a low density of collagen fibers. We should focus the existence of spindle cells similar to fibroblast with small and thin nucleus and scant cytoplasm. The limit between the intima and the media is evident. Muscular intimal atrophy and fibrosis around the muscular fascicules from the medial layer are visible. H-E.

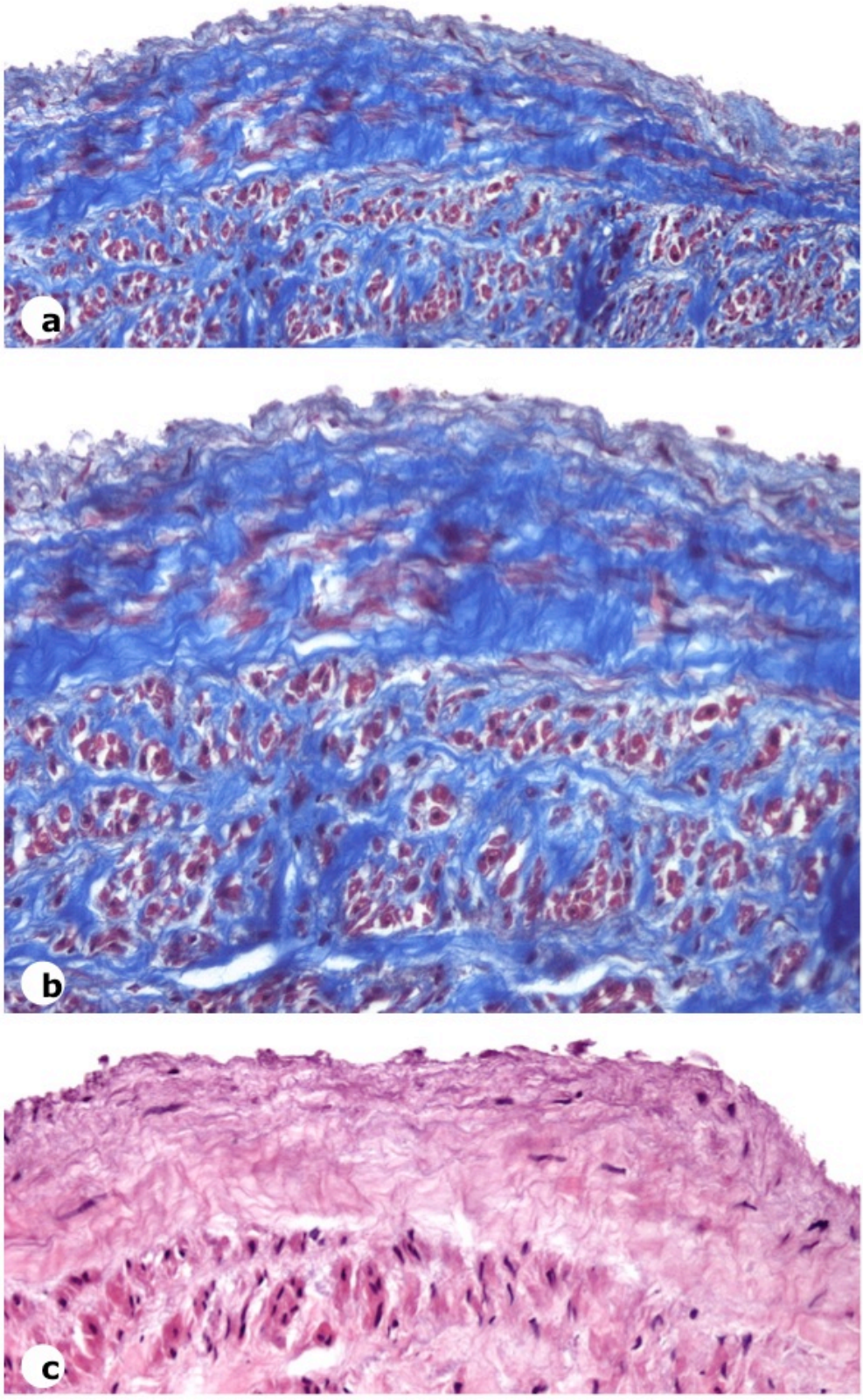
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Fig. 11. Smooth muscle alpha actin (SMA) expression in varicose vein wall

a: Panoramic image from a transverse section in the proximal segment from intense varicose lesions. It is seen a huge fibrosis at the intimal layer, although the lesion grade is different at each vascular hemisection. In the most thickness intimal areas can be observed an accumulation of SMA+ in SMCs. surrounded by connective tissue. In areas with less intimal lesions, the medial layer is former by multiple circumferential layers of SMCs. However, at the areas with intense intimal thickness, the SMCs loss their circular disposition, and are separated by an increase of connective tissue.

b: Distal segment of vein with a normal intima and an evident subintimal tissue with longitudinal SMCs surrounded by loose connective tissue. The medial layer is thickness and the leiomyocytes have a heavy expression of SMA.

c: It is observed an intimal thickening at the proximal segment from a saphenous vein. The connective tissue is dense with hypertrophic SMA+ fibers, but the majority of intimal cells show poor immunostain. In the medial layer, the interstice is higher due to the deposition of irregular connective tissue.

d: The image show another area from the same vein. It is observed several groups of SMCs with intense SMA immunohistochemistry reaction. Only at the subendothelial area are observed undifferentiated cells with low density of SMA. The leiomyocytes from the medial layer are bigger, although the intensity from the SMA immunoexpression is smaller than at the proliferating cells views in the surface of the intimal layer.

e: The image show a varicose nodule at the intima and at the superficial medial layer from the proximal level of a varicose vein. The intima has two SMA+ cell populations. At the bottom, the SMA+ cells are leiomyocytes cut transverse. At the superficial level, the SMA+ cells are smaller and they are grouped together at nest, but also few of them are isolated in the interior of the intimal ECM.

f: Detail from the medial layer in an area with a big intimal thickening. The muscular fibres have a widely thickness. The muscular fascicules are irregular, small and separated by big bands of connective tissue.

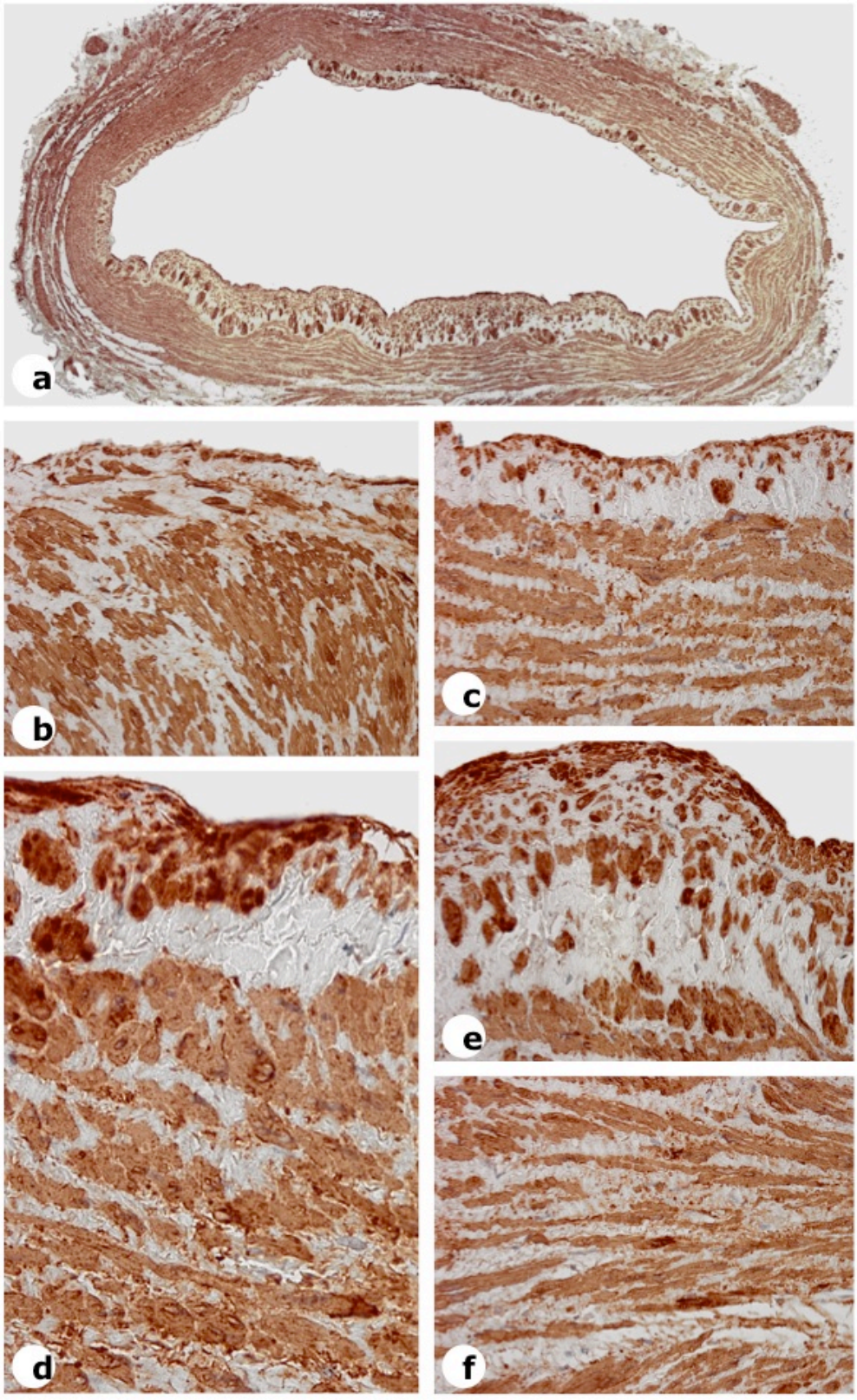
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Fig. 12. Vimentin expression from the wall of a saphenous vein with varicose lesions

a: It exists a progressive fibrosis at the proximal segment from the saphenous veins. The nodule of intimal thickness contains numerous cells with small cytoplasm and an intense vimentin expression. These cells are surrounded by ECM with low collagen fibers deposits. The limit between the media and the intima is clear and shows a thin internal elastic membrane. At the medial layer, the vimentin+ cells are poor and they are disposed rounded the fascicules of SMC.

b: It is observed a moderate thickening of the intima. The intima presents few vimentin+ spindle cells that they are more numerous at the subendothelial area. The medial layer has areas with initial fibrosis that contents a bigger number of vimentin+ fibroblast.

c: A huge proliferation of vimentin+ cells are situated in an area of initial fibrosis, sited under the endothelium. The connective tissue from the medial layer is not very abundant with presents a limited number of vimentin+ cells.

d: An intimal nodule in an area with intense intimal fibrosis is shown. The immunostain demonstrated the existence of transversal fascicules of vimentin+ cells. At the superficial level exist isolated cells with less expression of vimentin.

e: A nodule with intense fibrosis shows small fascicules of vimentin+ SMCs at the intima, surrounded by ECM. At the superior level of the intima, isolated and spindle cells with scant cytoplasm are observed. These undifferentiated cells are recognized by a vimentin stain lower than the intense vimentin stain of the cytoplasm of the endothelial cells.

f: The image included the interphase between the intima and the medial layer at the proximal venous segment. It is evident the bigger number of vimentin+ cells in the medial layer fibrotic tissue. The intima presents perpendicular fascicules of vimentin+ cells.

g: The image have a huge intimal thickness that contains an intense proliferation of cells with scant cytoplasm that showed an irregular vimentin stain distribution into their cytoplasm. Around these cells, thin bands of ECM are deposited. At most of the areas, the endothelial cells have been disappeared and substituted by fibrous tissue.

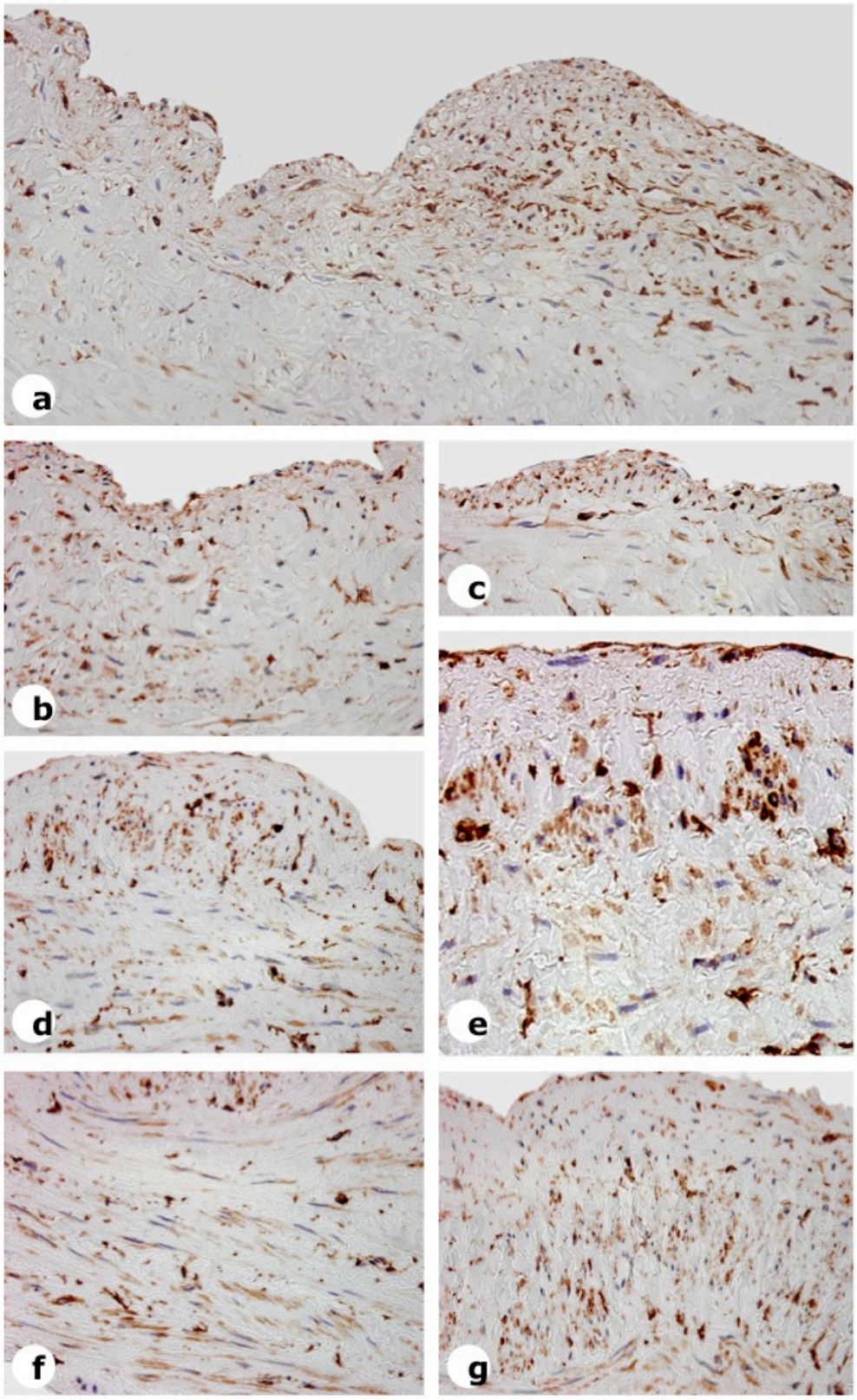
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Fig. 13. Ultramicrotome thin slices from saphenous vein wall with varicose lesions

a: We show a panoramic transversal section from a varicose proximal vein segment. At the intimal layer can be seen an intense thickening that cause an irregular surface of the vein lumen. The circumferential fascicules from the medial layer have a different size and an evident interstitial fibrosis. Toluidine blue.

b: Detail from before image. The intimal cells are distributed in two stratums. The bottom one constituted by cut transverse leiomyocytes. The other stratum has more collagenous tissue, and the cells are isolated, with showed oval or spindle shape and scant cytoplasm. The vein is tortuous, and show irregular fascicules of SMCs. Toluidine blue.

c: Section from a saphenous veins with an intense intimal thickening. The superior level of the intima has spindle cells surrounded by an extensive ECM, instead at bottom of the intimal layer where the SMCs are surrounded by less fibrous deposition. At the medial vein layer, fibrosis and atrophy from the leiomyocytes are evident. Toluidine blue.

d & e: At another field from the previous same vein sample with intense varicose lesions, it is demonstrated the characteristic double stratification from the intimal thickening. Moreover, the endothelium has disappeared and the intima contained numerous cells with hypertrophic nucleus and virtual cytoplasm surrounded in an amorphous loose ECM, associated to short and immature elastic fibers. Toluidine blue.

f & g: At an advanced stage of intimal fibrosis, the fascicules of transverse SMCs from the intima are getting atrophic and their leiomyocytes are transformed in dedifferentiated spindle cells. Moreover, the ECM and collagenization are bigger at this point. Toluidine blue.

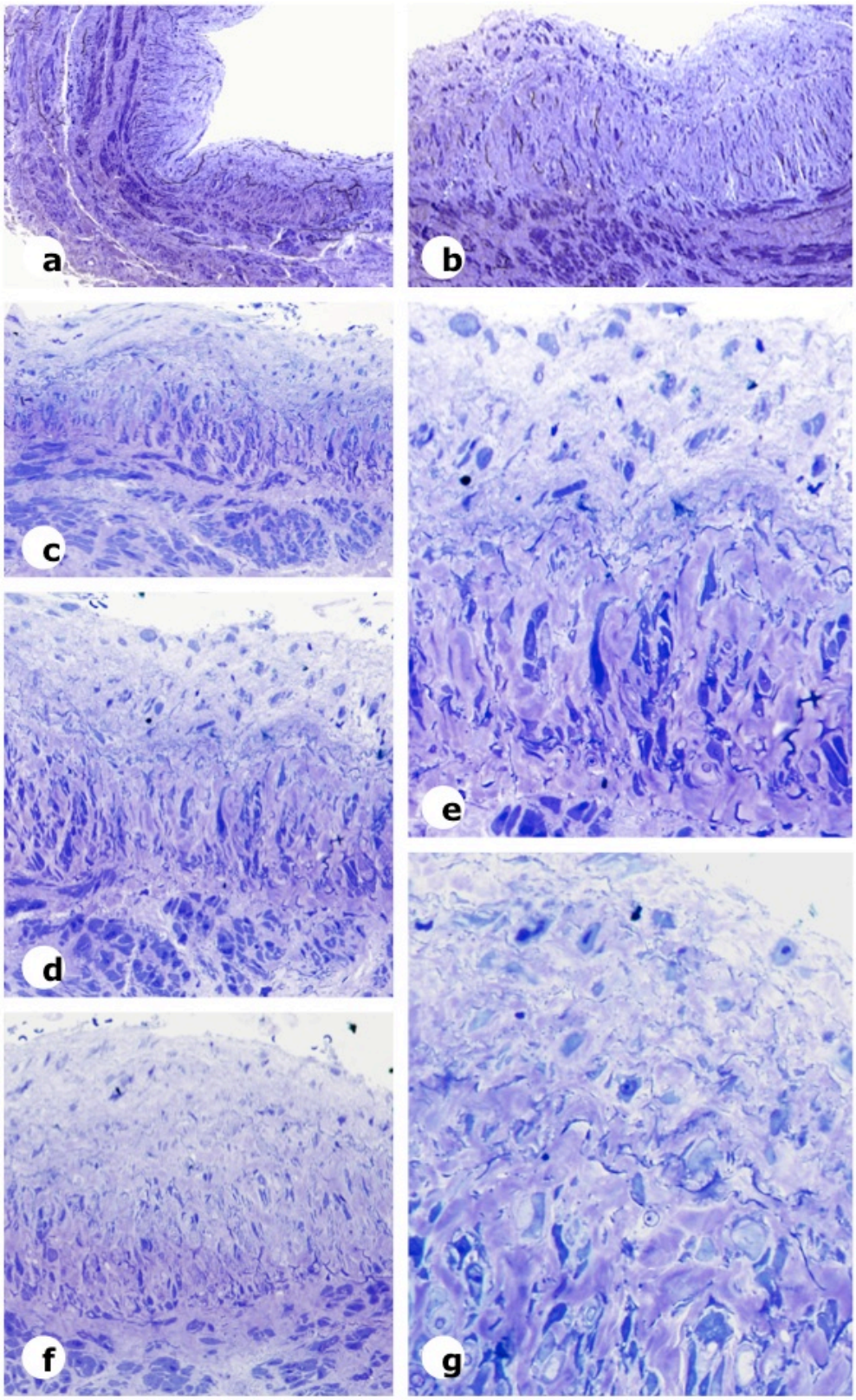
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Fig. 14. Ultrastructural changes in the saphenous vein with varicose lesions

a & b: In the intima, the internal elastic fiber is substituted by fibrous tissue and small, short and thin elastic fibers intermixed with the amorphous ECM. In some areas exist a progressive transformation from the intimal SMCs to oval or spindle dedifferentiates cells, some of this showed a hypertrophic nucleus. The luminal area is irregular and the endothelial cells are denuded. Toluidine blue.

c: Ultrastructural image from a varicose intimal lesion. At the bottom of the image has be seen a flexuous elastic membrane characterized by a central core of amorphous material with low electronic density. Also, some small spindle cells with circular or elongated nucleus and heterochromatic material can be seen under the karyotheca. The cytoplasm of this cells is dense and fibrillary.

d: This ultrastructural image shown the intimal surface where ECM deposition rounded the irregular collagen fibers. Moreover, we can observe two intimal cells with virtual cytoplasm and development of cytoplasmic prolongations that are surrounded by ECM.

e: This image contains a fragment of a cell in the intimal layer. The nucleus is hyperchromatic and with and showed an irregular shape. The cytoplasm is wide, with a polygonal shape and contains round mitochondria and fascicles of intermediate filaments. Additionally, it can be seen some dense disc blocking by myofilaments and desmosomes. Also, in the interstice near the cytoplasmic membrane, deposition of amorphous material, ECM and irregular elongated collagen fibers are demonstrated.

f: Can be observed the cytoplasm of the soma and the prolongations from a cell located into the intimal fibrotic tissue of a varicose vein. Inside the cytoplasm can be seen a big number of intermediate filaments. Also, can be seen dense discs with myofilaments and few desmosomes. The cell presents small vesicles, but no caveolae, so probably it is diagnosed as a myofibroblast. Near to the external lamella of the cytoplasmic membrane, an electrodense material that gets polymer to create isolated thin and short collagen fibers, surrounded by ECM are identified.

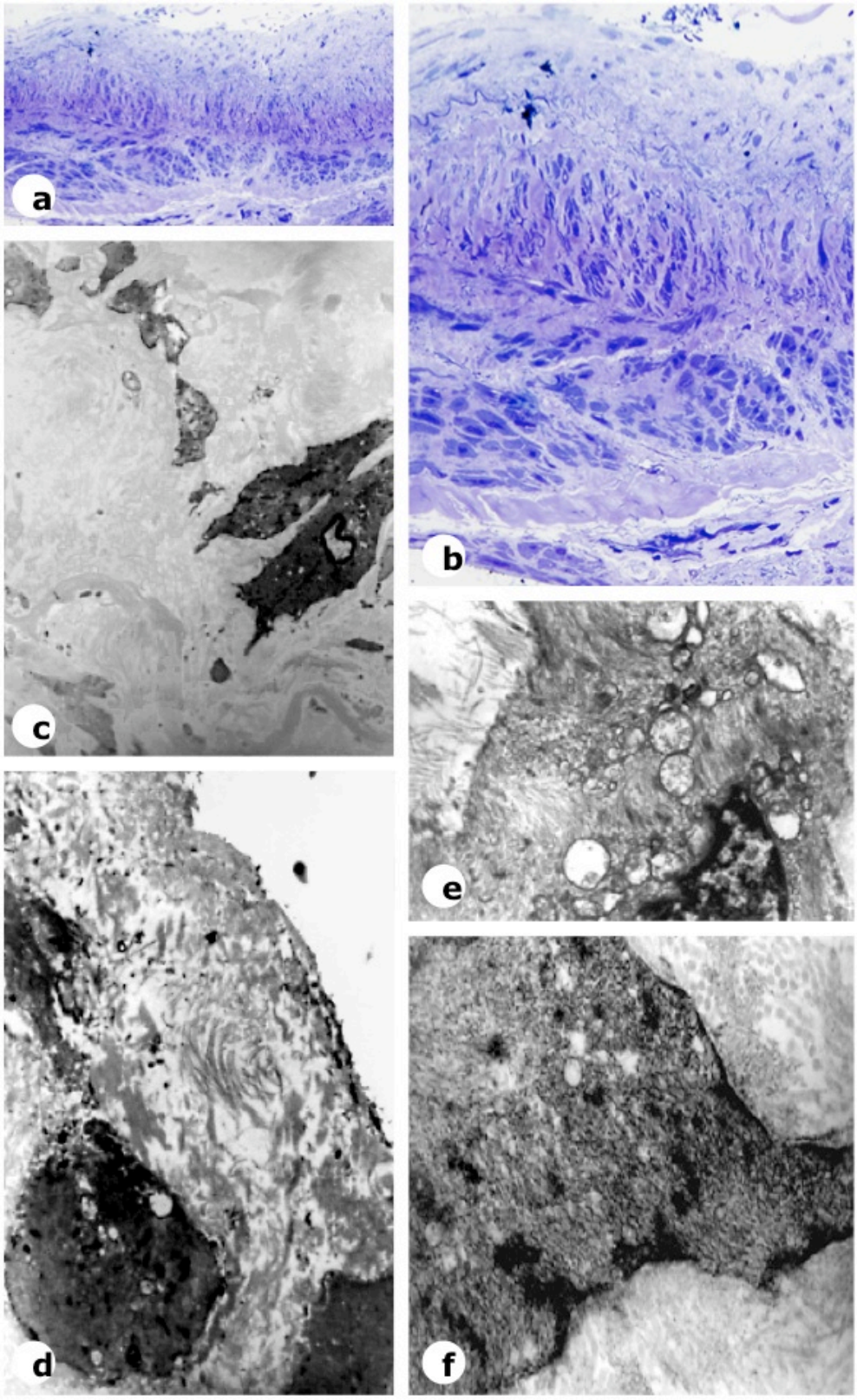
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Fig. 15. Study confocal microscopy of intimal lesions at the proximal venous segment with moderate or intense varicose lesions

a: The moderate fibrosis of the intima is characterized by a superficial layer with a proliferation of myointimal cells. At the deeper layer, an intense deposit of ECM and scant fibroblasts are demonstrated. H-E.

b: This photo is done in same histological area from the before image, but with higher birefringence, due to show the internal elastic fiber. H-E.

c: The rise of intima is associated to a proliferation of myofibroblast and bands of collagenous fibrous tissue deposits into lamina propria. This image is from the proximal surgical border of the superior segment from a varicose vein. This superior segment has also been study confocal microscopy. H-E.

d: Another field from the before area. Widely distribution of connective tissue at the intima and a proliferation of dedifferentiated myofibroblast are showed. The luminal surface present gaps due to loss of endothelium. Masson's trichrome.

e: Confocal microscopy study of endovascular surface of the proximal venous segment adjacent to before image. We merge images from z axis due to *in toto* at the superior level (80-90 μm deep) from the intimal thickening. The elastic fibers are short and thin. It can also be observed the great deposition of a granular elastic material with an intense green autofluorescence with the laser (λ=488).

f: Same field at the before image visualize by laser incidence proreflexion to show connective tissue. The thickening intima shown small collagen fibers, with an irregular distribution.

g: The nuclear stain of same before tissue sample demonstrated the existence of numerous small nucleus at the fibrous intima. Most of the nucleus has oval shape, but some of the enlargement nucleus is interpreted as intimal myofibroblast. DAPI stain.

h: Merge from the three previous images where it is shown the intimal disposition of the elastic material, collagen fibers and the ECM surrounded the myofibroblast and another muscular cells at the thickening intimal varicose lesion.

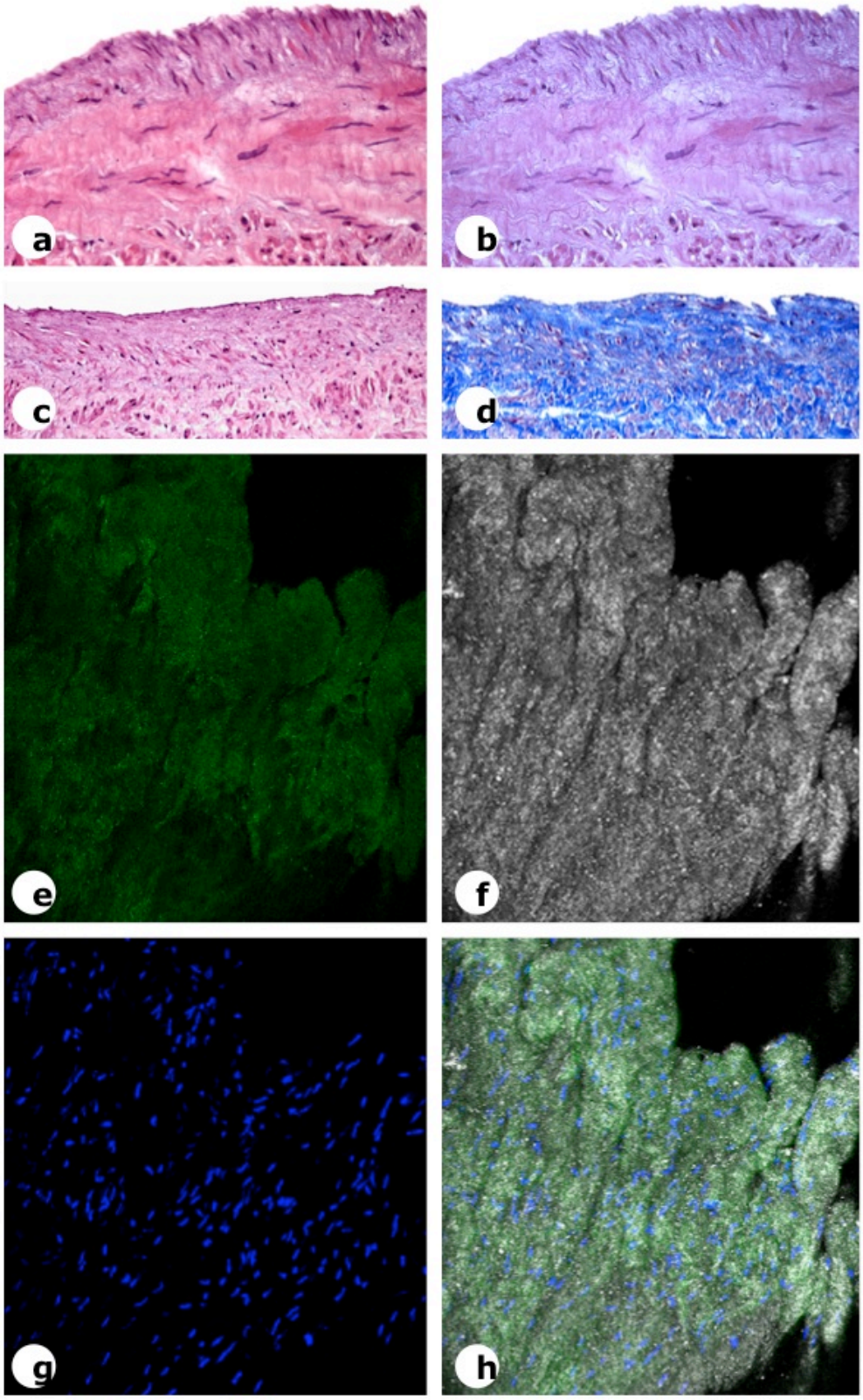
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Fig. 16. Study by confocal microscopy of intimal lesions at the distal venous segment with moderate varicose lesions

a: Panoramic image from the superior edge of the distal segment from a varicose vein. It can be seen an initial intimal thickening at the superficial area. At a deeper intimal area, the tissue showed strongly collagenization and can be identify dense fascicules of collagen fibers. At this area, the presences of spindle cells look like fibroblast are scant. H-E.

b: Detail from the before image. It is shown the connective tissue situated below the disappeared endothelium. The ECM is abundant and it surrounded the immature myofibroblast. At the deep intimal area the collagenization is bigger. H-E.

c: Photo taken at a close field from the before image. A loose connective tissue with abundant ECM is shown. Most cells are undifferentiated, but some present a spindle nucleus and a fibrillary cytoplasm, than look like myofibroblast. At the deepest area, the intima has a more intense collagenization, with thick collagen fibers in an irregular disposition. This fibrous tissue also has isolated SMCs of circular disposition. Masson's trichrome.

d: Confocal microscopy study of *in toto* intimal surface of the distal venous segment adjacent to image before. We merge images from Z axis at the superior level (80-90 µm deep) from the intimal thickening, with an abundant deposition of elastic material. It can be seen few small, short elastic fibers with spiral shape with an intense green autofluorescence observed in confocal laser (λ=488).

e: The reflection of the laser light at the same microscopic field allow to observe the deposition of thin collagen fibers, randomly distributed, into the intima. The darker areas correspond to folds of the intimal surface due to the circular normal shape of the vein.

f: A big proliferation of cells is found. The nucleus is small and close between them with a limited interstitial tissue. Most of these cells are fibroblast and myofibroblast. DAPI stain.

g: Merge image from elastic autofluorescence, light ray reflection and DAPI see in the three previous images that demonstrated the intense connective tissue deposition and collagen fascicules that surrounded the elastic material.

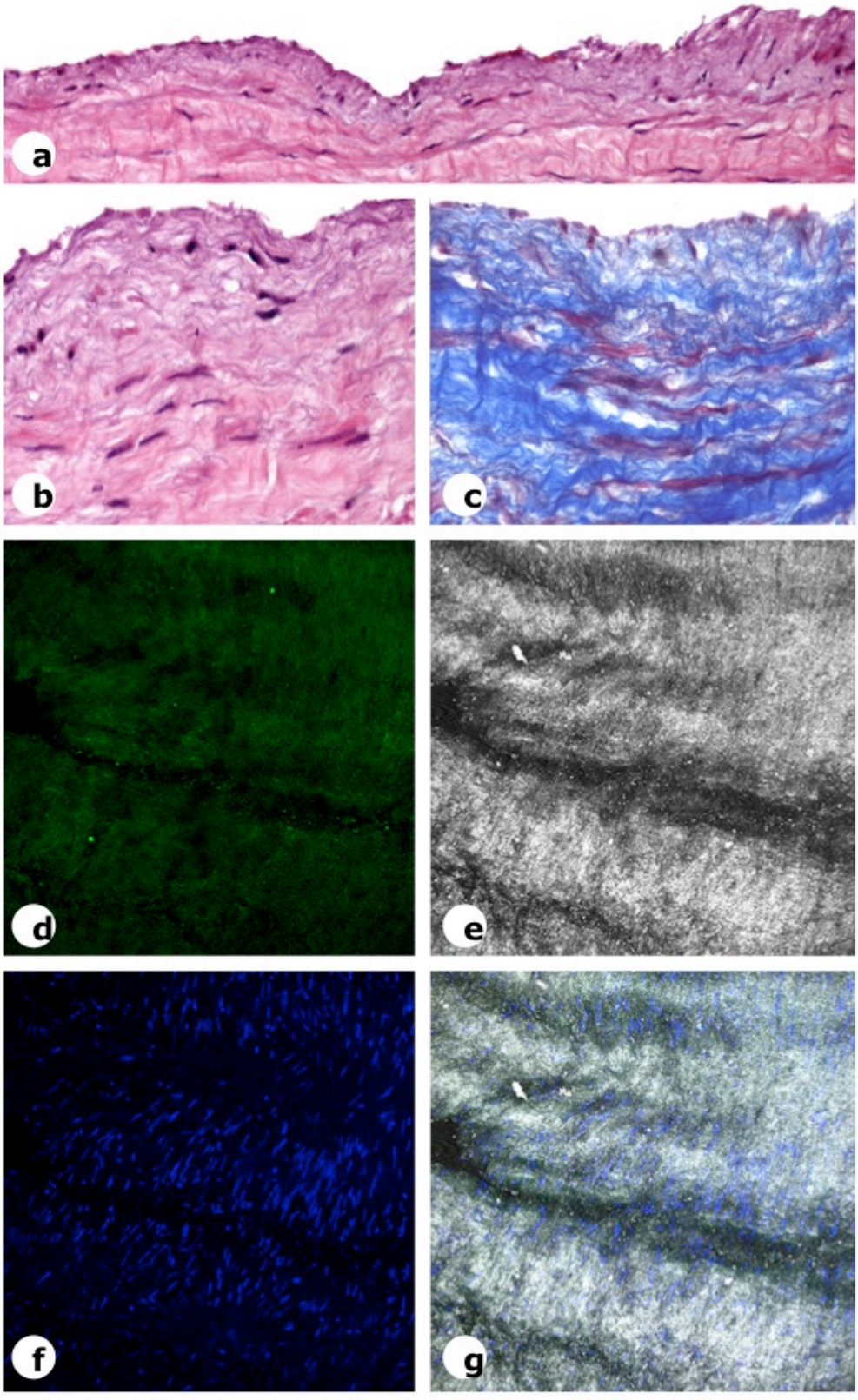
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Fig. 17. Confocal study of a Z axis of the intimal surface from the same distal segment image before

a: Autofluorescence image that represents the distribution of elastic material at the 14th Z axis at the same previous segment studied. The normal vein curvature determined the observation of two confocal segments. The elastic material is dust shape and it has an irregular distribution. Some of the areas with intimal fibrosis lack of elastin deposition.

b: The laser light reflection at the same field previously studied permit to see the strong deposition of dense amorphous connective tissue at intimal fibrosis. Additionally, it has been observed small collagen fibers with a transverse and oblique cut. Also, areas with less density of connective tissue possibly correspond to edematous ECM.

c: This confocal section has a limited number of round or spindle nucleus at the intimal fibrous tissue. DAPI stain.

d: Merge image from elastic autofluorescence, light ray reflection and DAPI at the 14th Z axis. This image corresponds to evident fibrosis of the intima layer secondary to varicose disease.

e & f: Merge image from elastic autofluorescence, light ray reflection and DAPI at the 14th Z axis. Image has been rotated to a plan perpendicular to the luminal surface. The image also represents histological structure from two randomly perpendicular plans respect the intimal surface view. So, it is possible to see at the left bottom and at the right bottom an image of the intimal fibrosis. At both plans the deposition of connective tissue is predominant respect to elastic fibers and elastic material deposition.

g & h: Histological border close to the superficial surface adjacent to the same intimal fibrosis plaque before studied *in toto* by confocal microscopy. Both images showed an initial intimal varicose lesion characterized by collagen deposition and rise of ECM. The cell proliferation in the intima is low. **g:** Masson's trichrome. **h:** H-E.

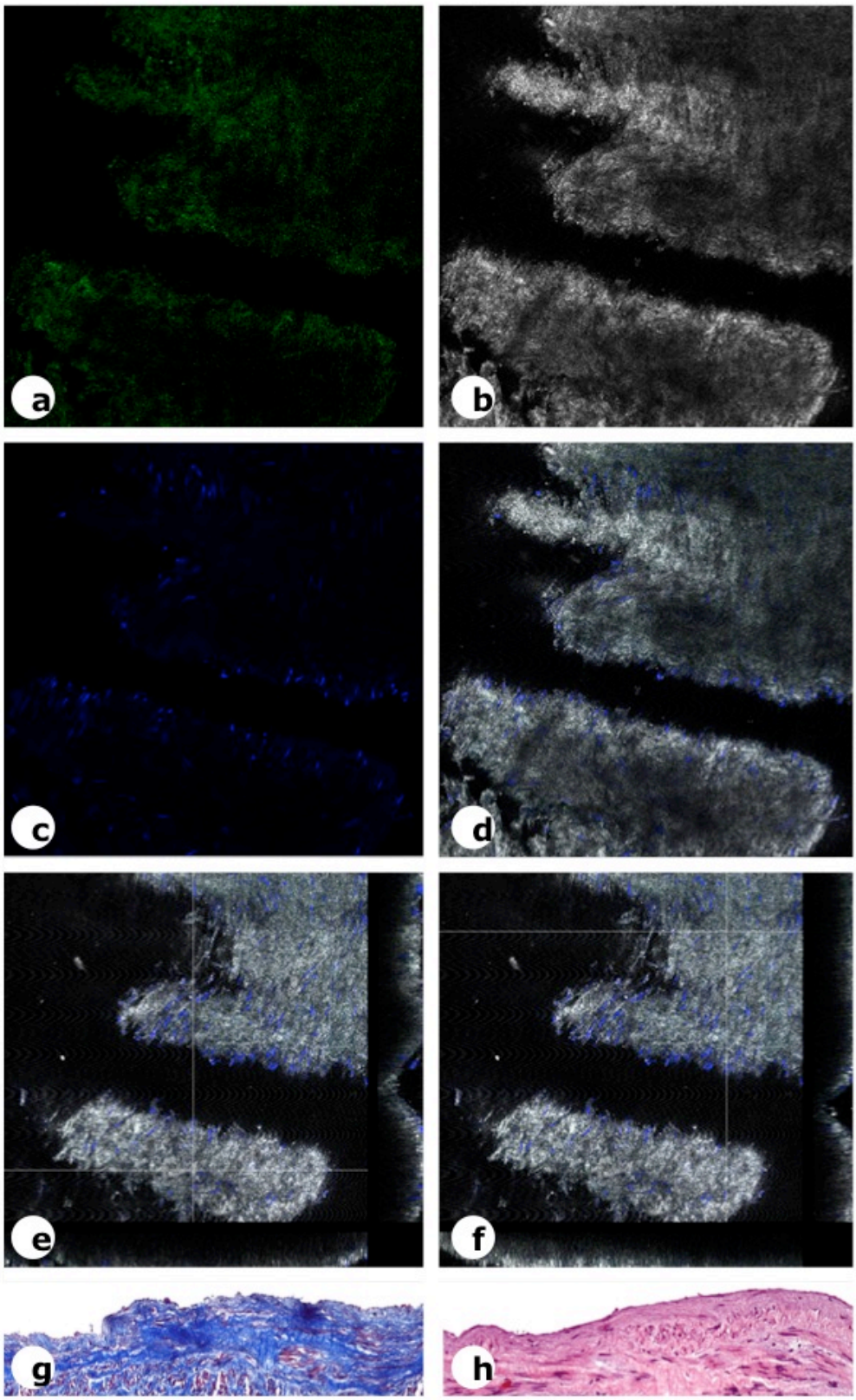
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Fig. 18. Expression of collagen type III at the proximal segment from varicose veins

a: Panoramic view of transverse section from a saphenous vein. The immunoexpresion of collagen type III is widely spread all over the vascular wall, but the expression is higher at the intimal layer.

b: At high magnification we can identify an intense anti-collagen type III reaction at the subendothelial intimal tissue, that constitute the venous lamina propria. In the central area of intima, the immunoexpresion is low, and the presence of intimal SMC determinate areas with pale color, surrounded by a lower deposit of collagen type III.

c: Detail from the superior area of before image.

d: Detail from the central area of same vein. At the central area of the intima, we can see the presence of SMC, that no express collagen type III, surrounded by a tenuous expression of collagen type III in the connective interstitial tissue.

e: Big magnification of the intimal layer. At the medial layer, we can see the presence of SMC surrounded by collagen type III.

f: Detail from the inferior area of same vein. In this transverse section we can easily distingue the intimal layer with a higher expression of collagen type III and a perpendicular disposition of the cells. The medial layer shows the typical circular disposition of the SMCs. intermixed with interstitial connective tissue, that contain evident deposit of collagen type III. This expression is related to initium of medial fibrosis, also frequently found in the media of varicose veins.

g: Transversal section of varicose vein with huge intimal fibrosis in the lower section. A scant fibrosis at the superficial section of the vein.

h: Detail of g. The lower section has a great intimal thickening with an intense collegen III stain. The superficial section has a moderate fibrosis.

i: Detail of the upper hemisection from photo g. The intimal layer has a intimal layer with a scant stain for collagen III due to the scant fibrosis.

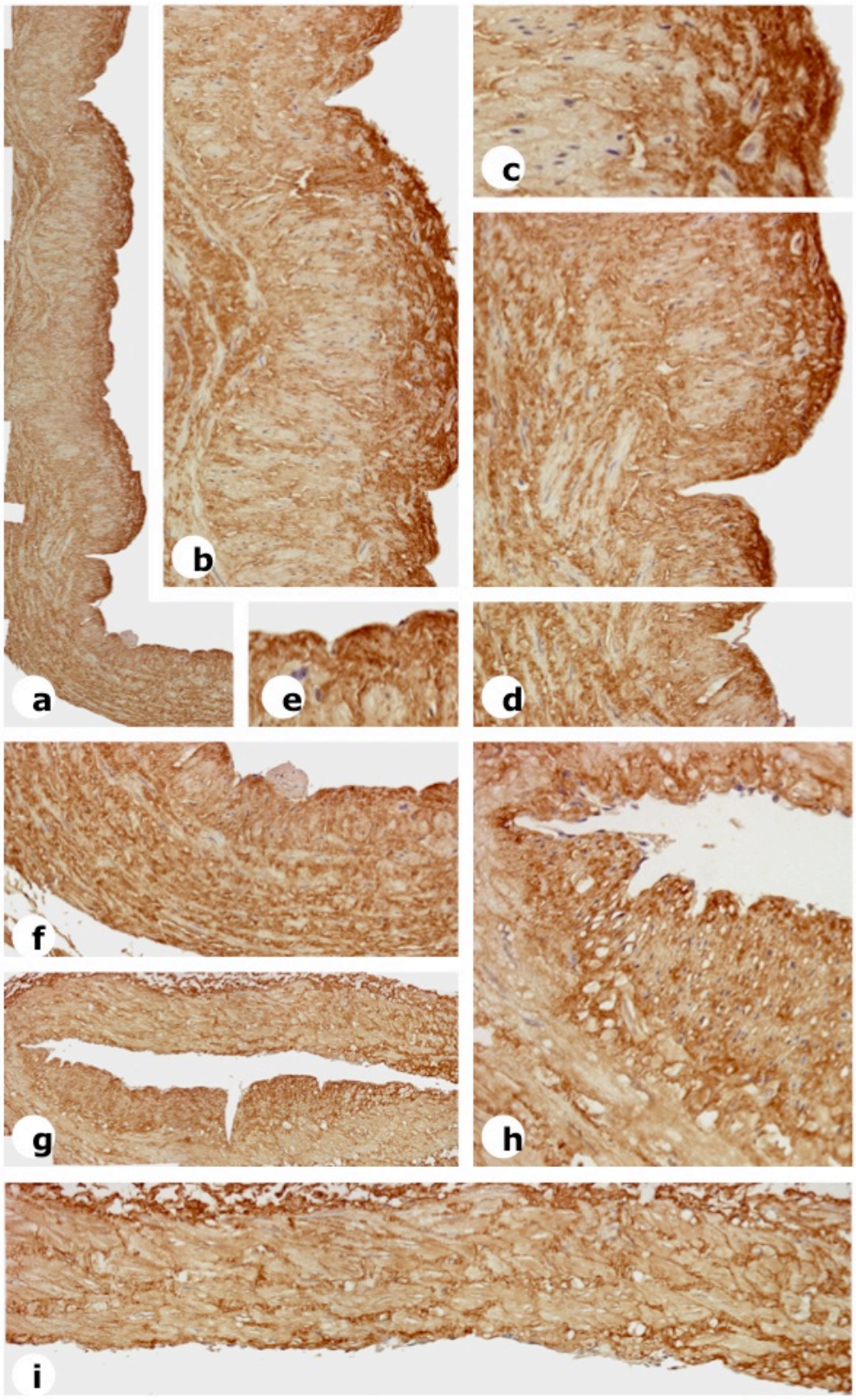
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Fig. 19. Expression of collagen type III at the distal segment from a varicose vein

a: Transverse section from the distal segment of a varicose vein. At the lumen of the photo we can see the intimal layer with an intense immunostain for collagen type III. The medial layer has a lower intensity of immunostain.

b: Detail of before image. The magnification also permit demonstrated the minimal deposit of collagen type III in the virtual interstitial tissue intermixed to the circumferential or oblique dispositions of SMCs. in the normal medial layer of distal vein.

c &d: Strong collagen type III immunostain in the lamina propria of an initial varicose intimal lesion vein.

d: Detail from the XXX image. The intimal layer has an intense stain for collagen type III, and the ECM has substituted the subendotelial cells. The medial layer is former for circumferential layer of SMCs with their ellipsoidal nucleus.

e: The panoramic image has been obtained in other area of same distal varicose vein sample. The collagen type III has a higher presence at the intimal layer than at the medial layer.

f & g: The detail demonstrated an intense reaction collagen type III immunoreaction in the subendothelial layer. However the stain is lower in the connective tissue disposing between the initial hyperplastic SMCs founded in the intima, as well as in the medial saphenous vein layer.

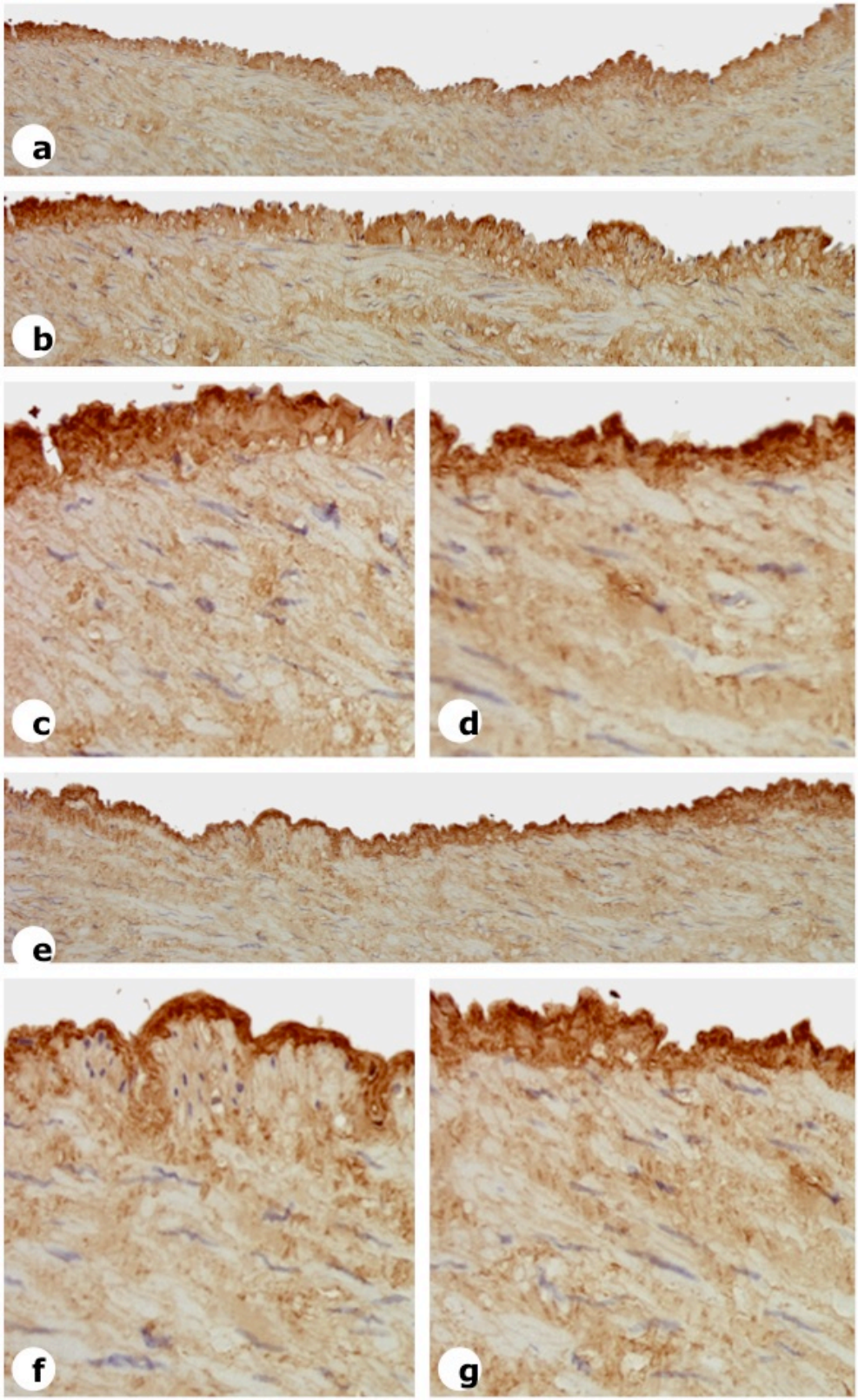
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Fig. 20. Expression of collagen type I at the proximal and distal segment from a varicose vein

a: The proximal segment of vein shows an intense thickened of the intimal layer characterized by a strong deposit of Collagen type I upper in the subendothelial lamina propria. The diffuse collagen type I deposit extends to the intestinal tissue that surrounded the SMC fascicles located at intima and media layers.

b: The intima of this proximal vein segment is extraordinary thickened and the immunostaining with collagen type I antibody demonstrates an intense fibrillar and diffuse pattern. The intensity of immunostaining is lower in the media layer.

c: Detail from the before image that demonstrated a strong band of immunoexpression of collagen type I at the lamina propria in vicinity to endothelial epithelium. The fibroblasts and myofibroblasts of the intima are embedded in the ECM and rich collagen type I connective tissue of the intima

d: This image obtained in the proximal segment of a saphenous vein shows the transition of a normal intima to an initial varicose lesion of the intima, characterized by hyperplasia of SMCs. and irregular deposits of collagen type I that intermixed with non-immunostaining ECM. Noted the high immunostaining seen in the interstitial connective tissue of medial layer.

e: This distal saphenous vein segment shows a soft and diffuse deposit of collagen type I.

f: Robust stain of collagen type I in lamina propria, if compared with the low expression in the deep intima. Noted an area with evident proliferation of intimal cells associated to the fibrillary distribution of collagen type I.

g: The grade of collagen type I expression is higher the intima surface and progressively decrease in the profound intima and in the vascular media.

h: Extensive fibrosis is found in the superficial area of the saphenous intima, due to strong imnunoexpression of collagen type I. However, the stain is lower in the profound area of this varicose vein.

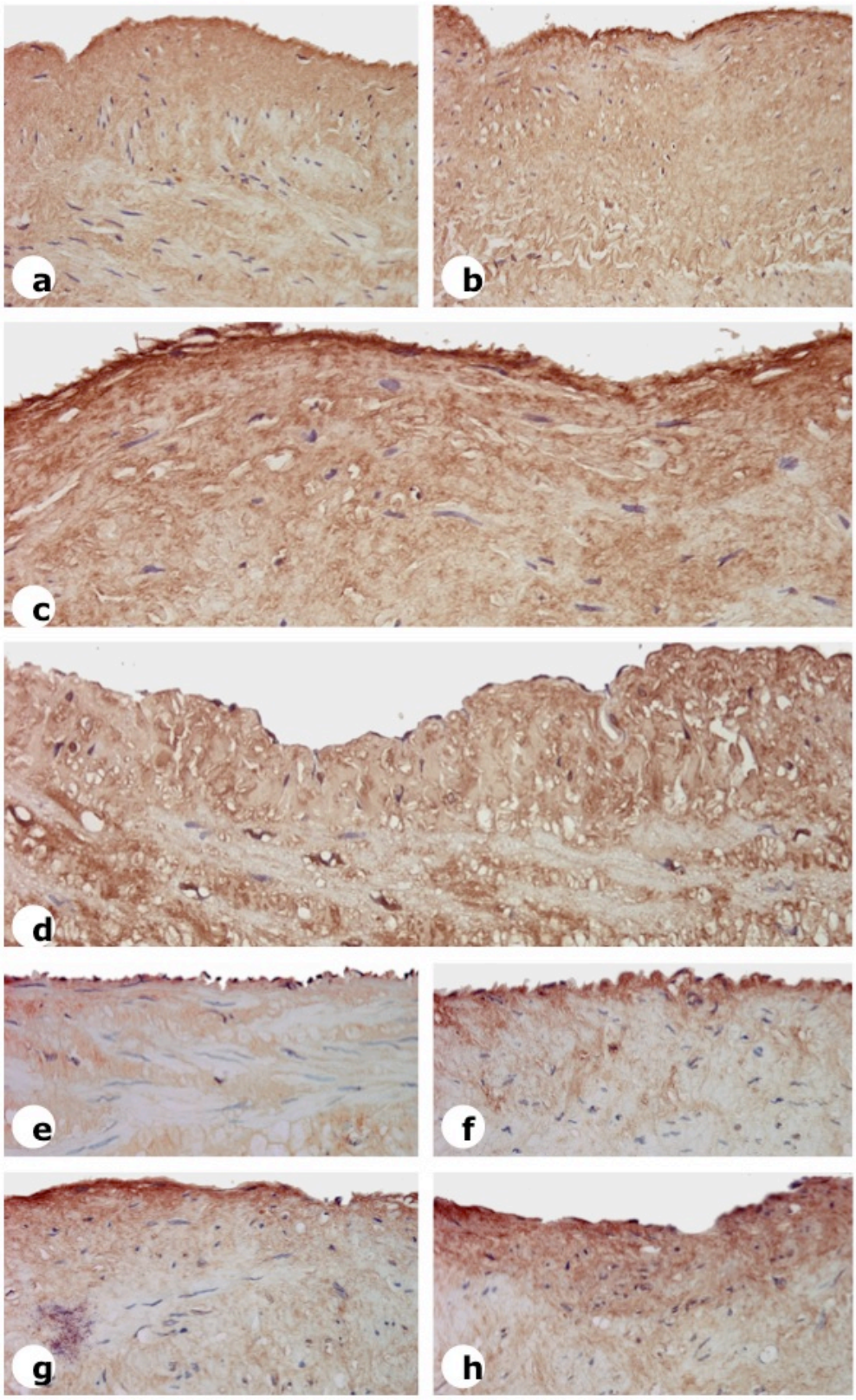
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Fig. 21. Elastic fibers from the wall of a proximal segment of saphenous vein with varicose lesions

a: Confocal microscopy observation of a transverse section from a saphenous varicose vein. The intense green color corresponds to elastic fibers that can be show by their autofluorescence activity.

b: Study of elastic fibers using confocal microscopy in other sample of intense intimal fibrosis in a varicose vein. The transverse vein section shows an intense green autofluorescence of the tortuous elastic fibers found in the interstitial connective tissue located in the media layer of vein wall. The thickened intima presents only few elastic fibers and moderate deposit of granular amorphous elastic material.

c: The varicose vein show a complete circumferential internal elastic lamina that delimited the intima to the media of saphenous vein. In the initial fibrosis of the intima, duplicated elastic fibers are observed. These elastic fibers are irregular and presents some gaps that are characteristics in the fibrotic process. Orcein stain.

d: In the another area of the intima, an evident rupture of the internal elastic lamina in seen. The intimal connective tissue contains deposit of small, granulated, amorphous elastic material, but not well established elastic fibers are demonstrated. Orcein stain

e, f & g: Higher intimal nodular fibrosis with partial degeneration of internal elastic lamina and massive deposit of non-fibrillary elastic material in this intense intimal varicose lesion. The media of vein wall showed scant sorted elastic fibers. Orcein stain

h & i: Central area of same nodule with identical intimal lesion. Orcein stain.

J & K: The contralateral extreme of the intimal nodule. The internal elastic lamina presents an evident disorganization with initial bifurcations. In the intima, associated to elastic amorphous material deposits multiple shorted degenerated elastic fibers are observed embedded in ECM of fibrotic tissue. Orcein stain.

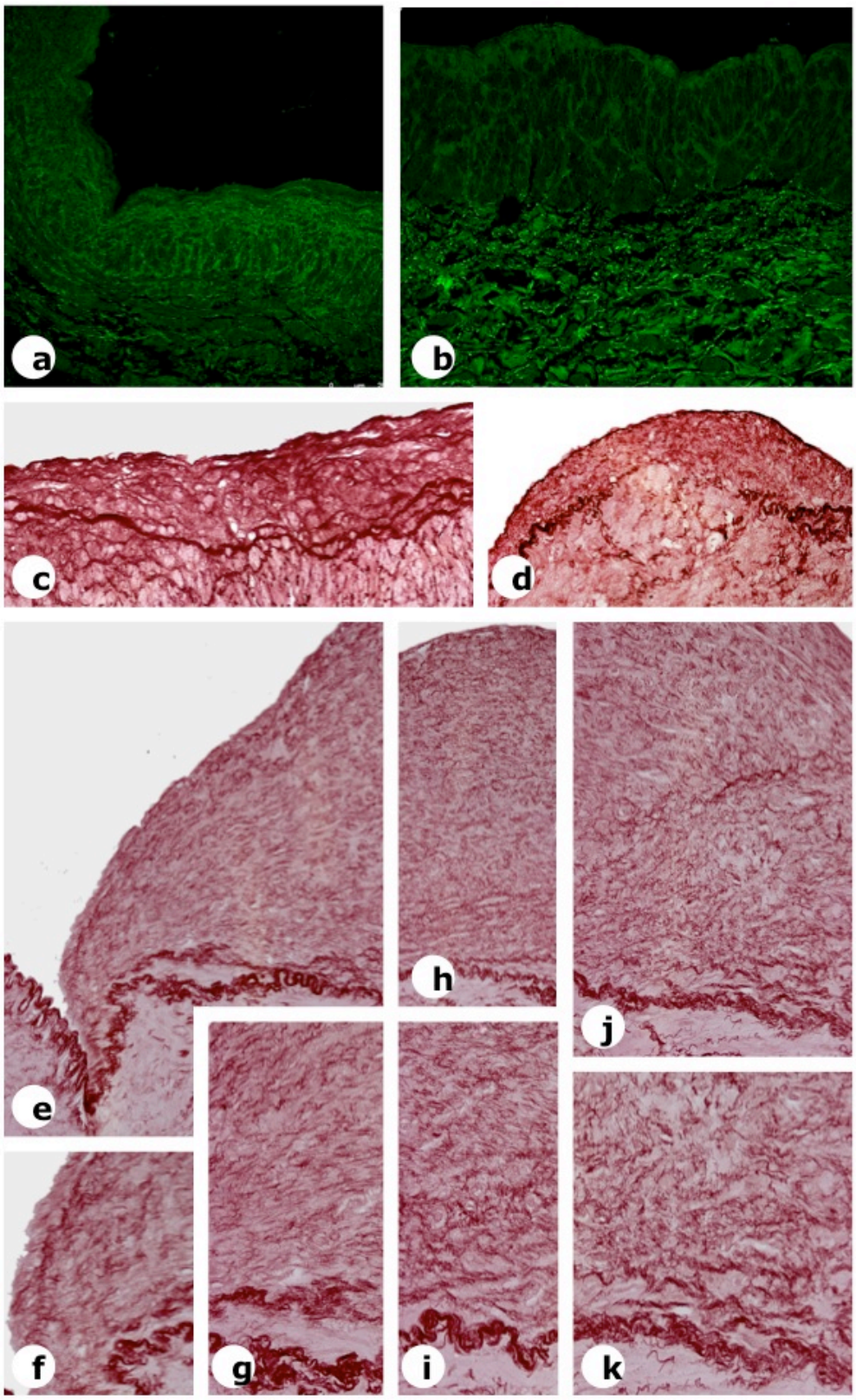
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Fig. 22. Elastic fibers from the wall of a distal segment of saphenous vein with initial varicose lesions in the intima

a: Transverse section from a distal segment of saphenous vein. The elastic autofluorescent permit to identify the three vein layers: intimal, medial and adventitial layers. The intima is virtual. The endothelial cells apparently are directly closely to elastic internal lamina.

b: An initial growth of the intima, an evident despotic of elastin fibers (green color) into the subendothelial lamina propria are found. This new elastic fibers are thin and irregular.

c, d & e: Different grade of initial varicose alterations. The normal intima progressively presents EMC deposits, reduplication of elastic internal lamina and a new formed intimal elastic fiber population. Short and scant elastic fibers deposits into the interstitial connective tissue that surround the circumferential fascicles of SMC are found. Orcein stain.

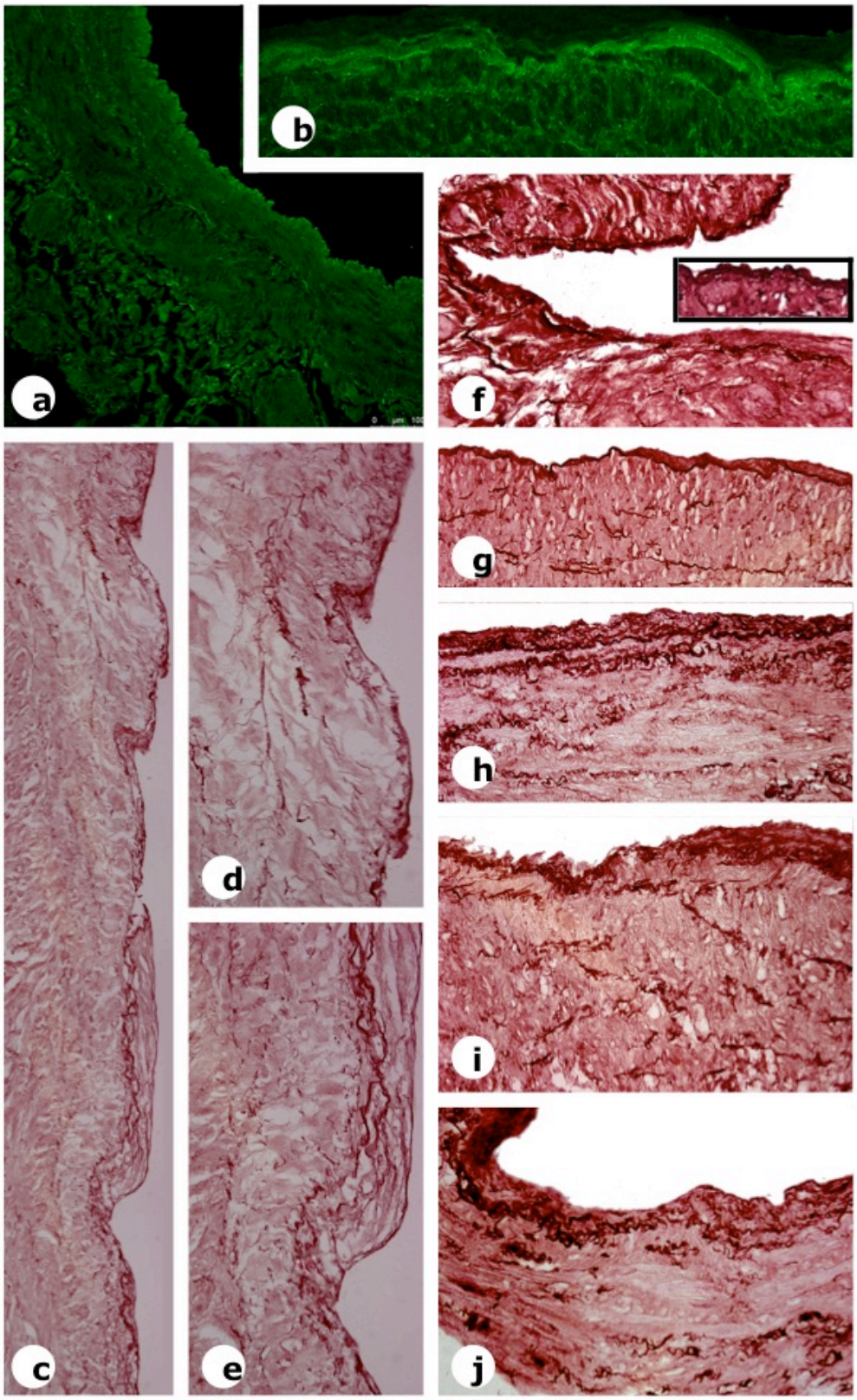
f: Normal distal segment of saphenous vein. Noted the direct transition to an initial progressively deposit of connective tissue and elastic amorphous material into the intima. The internal elastic lamina is normal. **Inset:** the lamina propria is virtual. Orcein stain.

g: Transition of normal intima to minimal intimal fibrosis without deposit of new training elastic fibers. Orcein stain.

h: Disorganization of the internal elastic lamina and the dust shape elastic fibers deposition. An increase of elastic fibers is observed in the medial layer of this distal vein segment.

i: Moderate thickening of the intima and rupture of internal elastic lamina. This nodule if still growing can produce a moderate partial stenosis of the vein lumen. In the intima, we can appreciate the different deposition of immature elastic fibers, characterized by the presence of multiple shorted elastic fibers embedded by connective tissue. Orcein stain.

j: Transmural varicose lesions. Disorganization of the internal elastic lamina and deposit of multiples elastic fibers into the intima were associated to atrophy of SMC fascicles, interstitial fibrosis and a degeneration of elastic fibers in the medial layer.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COLLAGEN TYPE I HISTOMETRY QUANTIFYING IN VENOUS INTIMA

Table 4 shown histometric data from the area occupied by collagen type I immunoreaction positive in relation to intimal total area of the vein. We considered three groups in relation to height of vein intimal layer.

Table 4. Collagen type I area.

Intimal height thickness (µm)	**Intimal total area** (µm^2)	**Collagen I area** (µm^2)	**Proportion collagen I area/total area**
1 to 39.9	21742 ± 8153	5374 ± 3088	0.24 ± 0.10
39.9 to 89.9	60923 ± 13890[a]	26032 ± 9577[a]	0.42 ± 0.10[a]
> 90	149382 ± 38622[a]	53194 ±14958[a]	0.35 ± 0.02

a: p<0.05 significant to before group

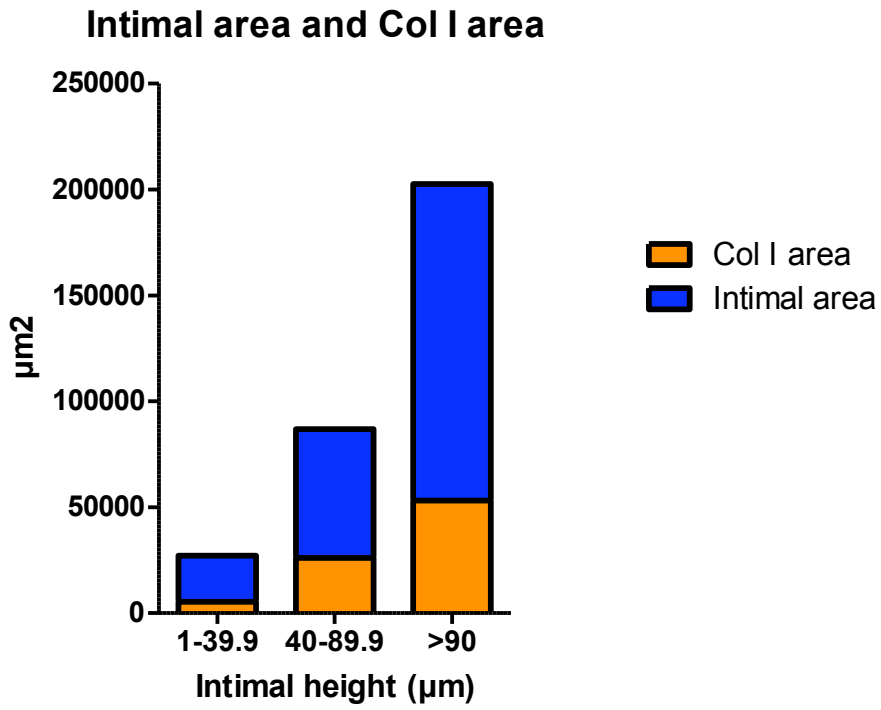


Figure 23. Total intimal area of the vein and collagen type I immunoexpresion at different intimal thickness. We considered three groups in relation to height of vein intimal layer.

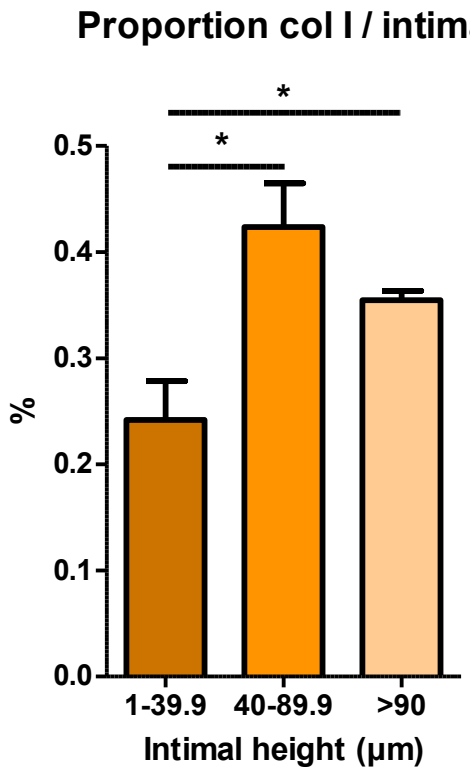


Figure 24. Proportion of collagen type I area to intimal total area. * p<0.05

COLAGEN TYPE III HISTOMETRY QUANTIFYING

Table 5 exhibit morphometric data about anti-collagen III immunoreaction positive in relation to intimal total area of the vein. We considered three groups in relation to height of vein intimal layer.

Table 5. Collagen type III area.

Intimal height thickness (µm)	Intimal total area (µm²)	Collagen III area (µm²)	Proportion collagen III area/total area
1 to 39.9	18019 ± 10465	8242 ± 6299	0.42 ± 0,15
39.9 to 89.9	65239 ± 17112[a]	28221 ± 11458[a]	0.43 ± 0,14
> 90	144236 ± 38680[a]	42221 ± 8731[a]	0.31 ± 0,09[a]

a: p<0.05 significant to before group

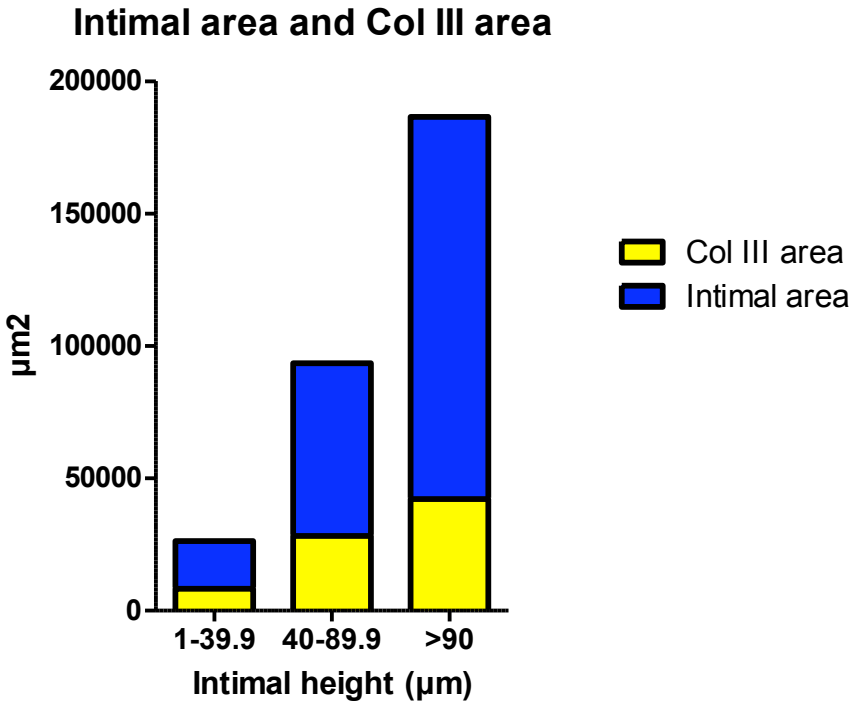


Figure 25. Total intimal area and collagen type III immunoexpresion at different intimal thickness.

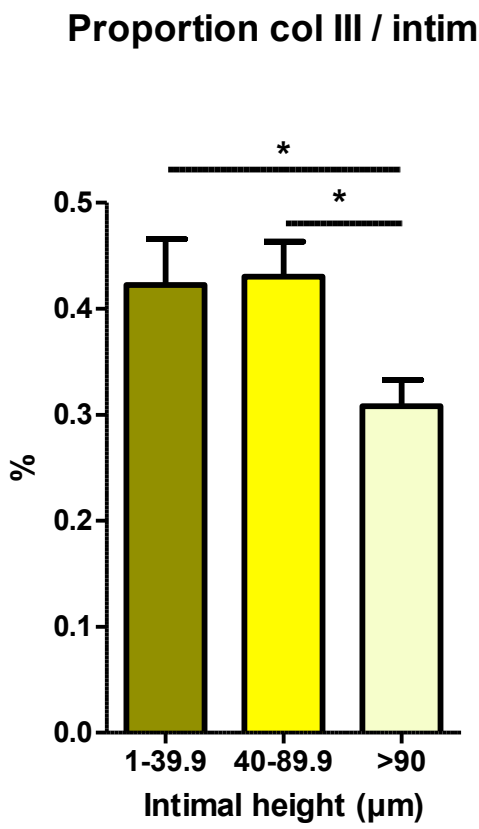


Figure 26. Proportion of collagen type III area to intimal total area. * p<0.05

SMA HISTOMETRY QUANTIFYING

Table 6 exhibit morphometric data about SMA immunoreaction positive in relation to intimal total area of the vein. We considered three groups in relation to height of vein intimal layer.

Table 6. SMA area.

Intimal height thickness (µm)	**Intimal total area** (µm^2)	**SMA area** (µm^2)	**Proportion SMA area/total area**
1 to 39.9	22097 ± 16113	5547 ± 4248	0.29 ± 0,10
39.9 to 89.9	68983 ± 8739[a]	15520 ± 7589[a]	0.22 ± 0,10[a]
> 90	168011 ± 39963[a]	25683 ± 4943[a]	0.16 ±0.03[a]

a: p<0.05 significant to before group

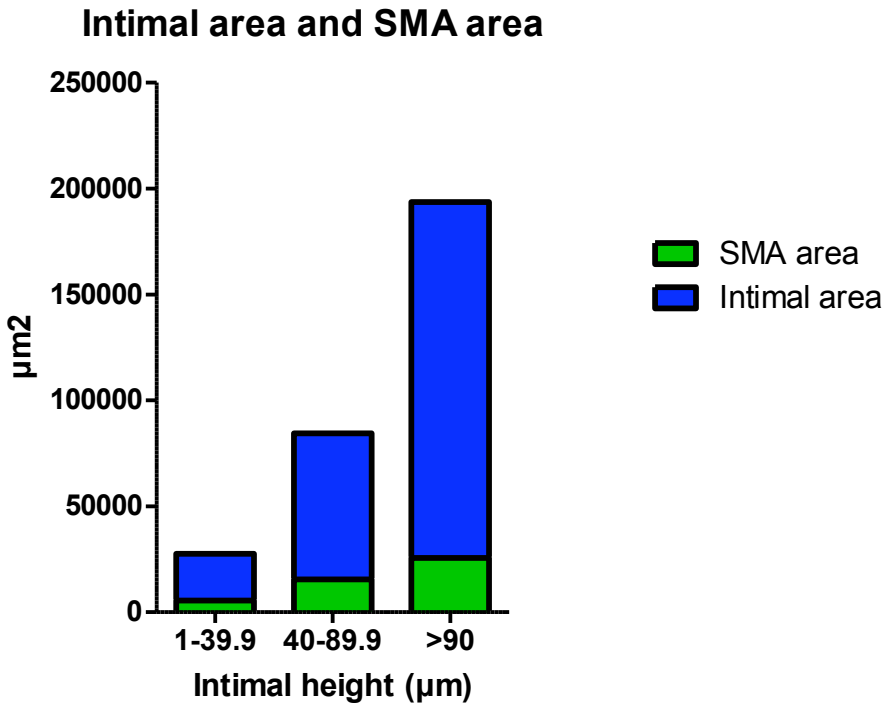


Figure 27. Total intimal area and SMA immunoexpresion at different intimal thickness.

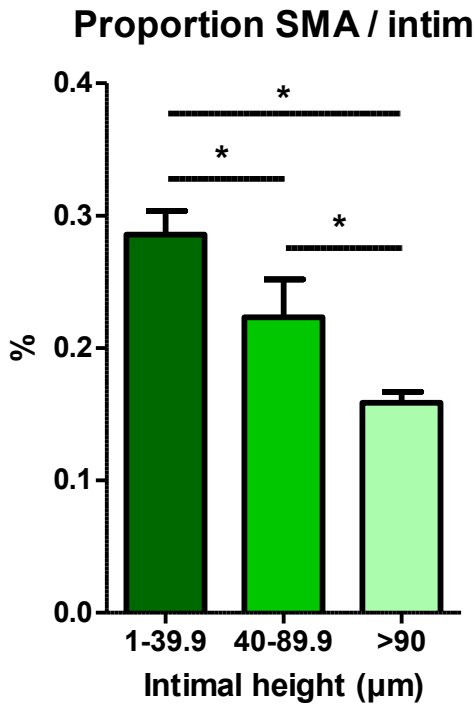


Figure 28. Proportion of SMA area to intimal total area. * p<0.05

ELASTIC FIBERS HISTOMETRY QUANTIFYING

Table 7 shown morphometric data from elastic fibers orcein +, total intimal area and proportion between elastic fibers area and the intimal area of the vein. We considered three groups in relation to height of vein intimal layer.

Table 7. Elastic fibers area.

Intimal height thickness (µm)	**Intimal total area** (μm^2)	**Elastic fibers area** (μm^2)	**Proportion elastic fibers area/total area**
1 to 39.9	20101 ± 12170	5993 ± 3209	0.32 ± 0,05
39.9 to 89.9	74692 ± 15364[a]	13892 ± 3066[a]	0.19 ± 0,04[a]
> 90	160574 ± 33499[a]	20252 ± 5538[a]	0.13 ± 0,04[a]

a: p<0.05 significant to before group

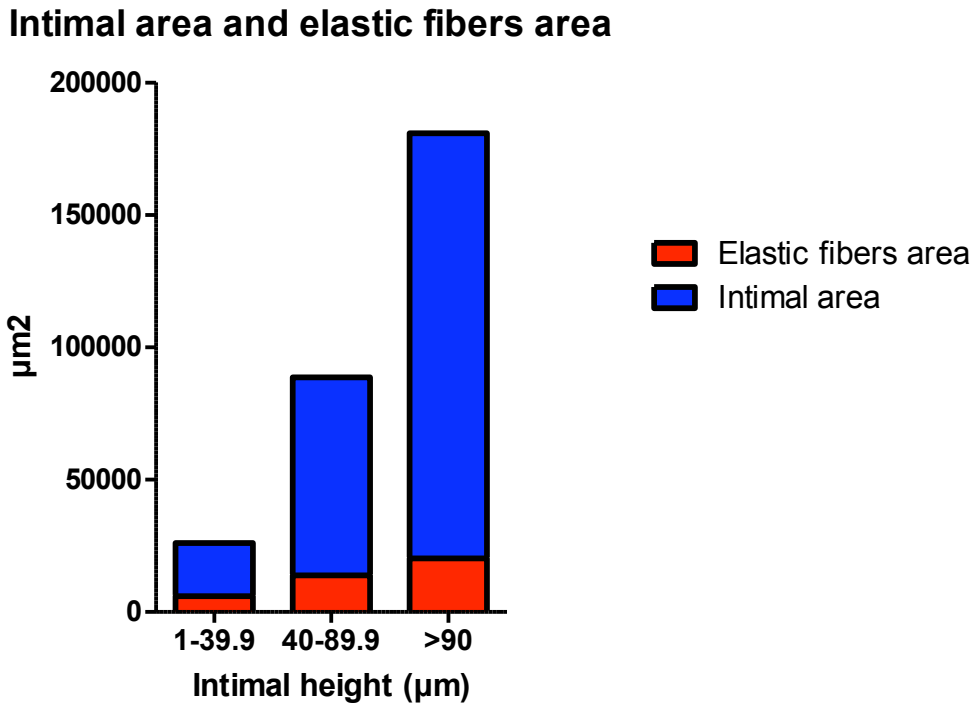


Figure 29. Total intimal area and elastic fibers orcein + at different intimal thickness.

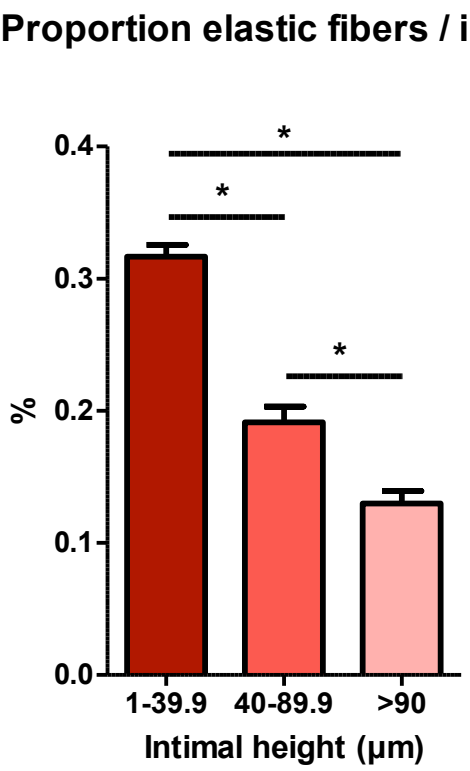


Figure 30. Proportion of elastic fibers area to intimal total area. * p<0.05

MOLECULAR QUANTIFICATION OF COLLAGEN TYPE I, COLLAGEN TYPE III, ELASTIN AND SMA IN PROXIMAL AND DISTAL SEGMENTS OF VARICOSE VEINS

The collagen type I, collagen type III and elastin mRNAs. were studied of the both proximal and distal saphenous vein segments. The data showed distal and proximal important differences between them venous segments.

The study of collagen type I, collagen type III and elastin, all of them implied at the synthesis of ECM are significantly upregulated at the proximal segment (Figure X). Also, the expression of SMA, not a strictly ECM gen, but very important for the mechanical function of the vein wall are significantly upregulated (Figure X).

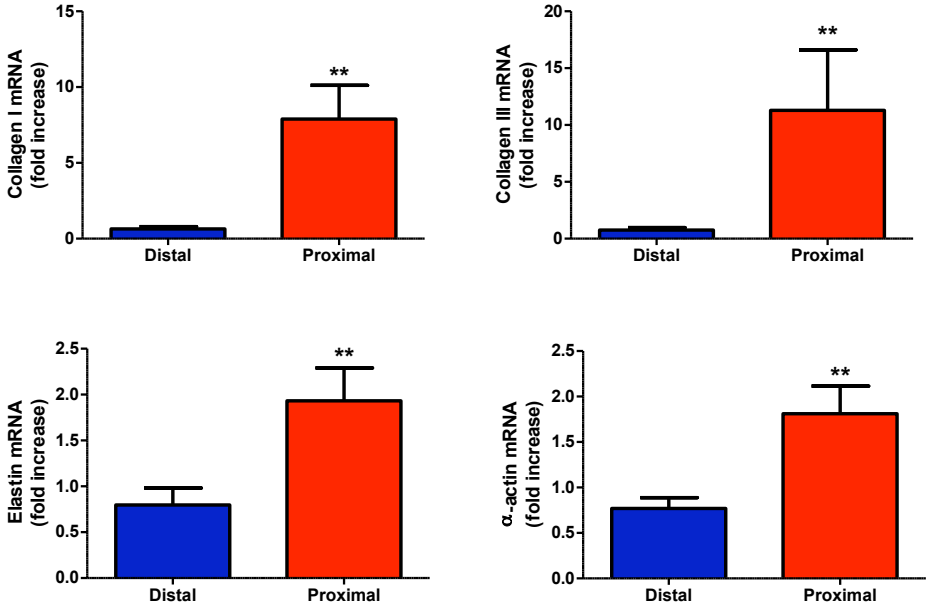
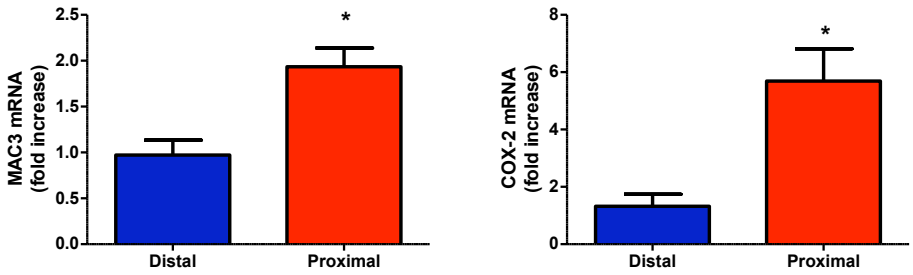


Figure 31. **a:** collagen type I mRNA levels, **b:** collagen type III mRNA levels, **c:** elastin mRNA levels and **d:** SMA mRNA levels. Data are expressed as mean ± SEM. **P<0.01 vs. distal segment.

MOLECULAR STUDY OF INFLAMMATORY AND ROS MECHANISMS RELATED TO VARICOSE LESIONS

When we studied genes with an inflammatory profile or related with ROS activity we show significant differences in the proximal and distal segments of varicose veins. The expression of MAC3, COX2 and NOX4 are significantly upregulated at the proximal segment (Figure X). Also the expression of mPGES looks upregulated, but the data don't show statistical differences. It was also studied the expression of NOX1, but we could not detect mRNA levels.



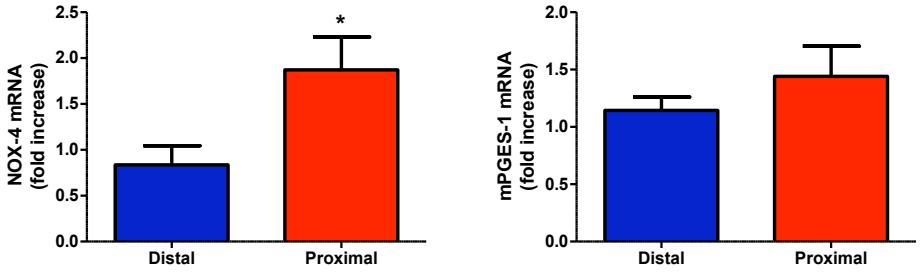


Figure 32. a: MAC3 mRNA levels, **b:** COX2 mRNA levels, **c:** NOX4 mRNA levels, **d:** mPGES mRNA levels. Data are expressed as mean ± SEM. *P<0.05 vs. distal segment.

In relation with ROS pathways, it was also studied the H_2O_2 production by AMPLEX-RED, due to NOX-4 is one of the enzymes implies at H_2O_2 production. Moreover, our data showed a significant increase production of H_2O_2 at the proximal level compared to the distal segment (Figure X).

Finally, we studied the NAPDH oxidase activity due to NADPH oxidase generates superoxide by transferring electrons from NADPH and can be implied at ROS stress. The NADPH oxidase activity was significantly higher at the proximal segment than at the distal segment (Figure X).

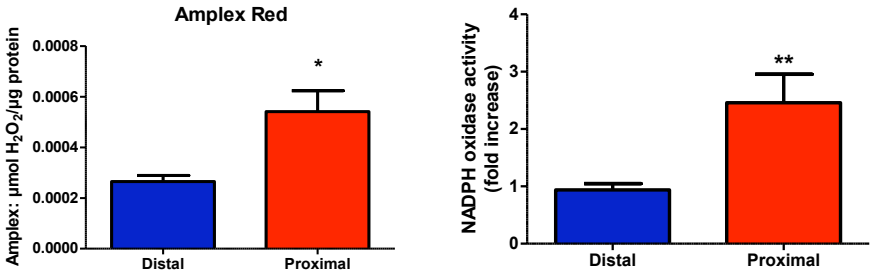


Figure 33. a: H_2O_2 production and **b:** NAPDH oxidase activity. Data are expressed as mean ± SEM. *P<0.05, **P<0.01 vs. distal segment.

DISCUSIÓN

La presente Tesis se centra en la valoración inmunohistoquímica, morfométrica y molecular de las mecanismos iniciales involucrados en el desarrollo de varices en la extremidad inferior. Estudios previos han señalado que muy probablemente la enfermedad varicosa sea el resultado de alteraciones primarias de la pared vascular y secundariamente aparezca una insuficiencia valvular, determinando ambos procesos trastornos hemodinámicos, con aumento de la presión hidrostática intraluminal debida al reflujo sanguíneo.[19] Se sabe que la alta presión sanguínea es responsable de los cabios morfológicos y funcionales de los vasos sanguíneos, de modo que la alta presión venosa causaría la formación de venas varicosas y de la ICV.[11]

Descripciones clásicas histológicas de las lesiones varicosas de la vena safena se han centrado en la existencia de engrosamiento de la íntima, y alteraciones de la capa media vascular. Estas lesiones se caracterizan por la presencia de hipertrofia o atrofia de las fibras musculares lisas de la capa media, además muy frecuentemente se produce en la capa media un incremento de ECM, una alteración de las fibras elásticas y una fibrosis intersticial; de modo que todos estos cambios determinan una alteración del tono venoso.[74,225,289]

En pacientes con enfermedad varicosa bien establecida, estudios clínicos y hemodinámicos de han demostrado que la formación de varices es mucho más frecuente y las lesiones son mucho más relevantes en el segmento proximal que en el segmento distal de la vena safena interna, segmento en el que los cambios hemodinámicos son menos importantes. Como bien sabemos, la principal fuente de reflujo en venas varicosas primarias en la unión safeno-femoral o la unión safeno-poplítea, así que el reflujo puede ser considerado como un cambio de vía de la sangre venosa desde el muslo hacia la parte inferior de la pierna.[227]

Si bien los cambios hemodinámicos y las lesiones morfológicas elementales de las varices están bien establecidas; sin embargo, en nuestro conocimiento, no existe un estudio sistematizado de comparación de las lesiones histológicas iníciales situadas en el segmento distal, con respecto a las lesiones bien establecidas de varices situadas en el segmento proximal. Por ello, en la presente Tesis se ha evaluado una serie de venas safena extirpadas en pacientes con IVC con lesiones de enfermedad varicosas no muy evolucionadas, pero en los que la flebectomía está clínicamente indicada (pacientes en estadio CEAP 2).[91]

En definitiva, este estudio no sólo tiene un interés académico, si no que, al definir los patrones moleculares y morfológicos de las lesiones varicosas ayudara en el diseño de nuevos estudios

clínicos, histopatológicos y moleculares *in vitro*. Cabe la posibilidad de que estas investigaciones permitan la identificación de posibles dianas terapéuticas que eviten o disminuyan el daño vascular, lo que redundará en un beneficio para el paciente al poder alterar el proceso evolutivo de la IVC y en una disminución de los costes sanitarios.

En el presente estudio, hemos comparado los progresivos cambios histológicos que experimenta la íntima vascular de la vena safena, desde los momentos iniciales con mínimo engrosamiento hasta las lesiones bien establecidas donde se producen engrosamientos intímales superiores a las 200 µm de espesor. Se excluyeron para este estudio aquellos casos en los que, además de la lesión intimal, aparecían evidentes alteraciones de la capa media vascular, incluidas áreas de fibrosis intersticial; de este modo al estar los cambios histológicos mayoritariamente en la íntima, se pudieron comparar los datos inmunohistoquímicos, histológicos y morfométricos con los cambios moleculares de la pared varicosa, y cotejar estos datos con el grado de engrosamiento intimal, esto es con las alteraciones de la ECM y de las células intimales de los segmentos varicosos. Además, para un conocimiento en mayor profundidad de la estructura y composición de la pared venosa varicosa se utilizo por primera vez en patología venosa una técnica basada en la refracción de las fibras de colágeno y la autofluorescencia de las fibras elásticas a través del microscopio confocal y así estudiar la disposición del colágeno y de las fibras elásticas.[236]

En el material estudiado hemos observado que, en los segmentos de pared de vena safena de apariencia macroscópica normal, el estudio histológico detallado permite visualizar áreas de íntima rigurosamente normal, revestidas por un endotelio plano, un tejido conjuntivo subendotelial y un lámina propia, donde el grosor de la capa íntima es mínimo; adyacentemente se pueden observar pequeñas irregularidades y engrosamientos intímales, caracterizados por un fino depósito de tejido conjuntivo entre el endotelio y la lámina elástica interna. En estas áreas con mínimos cambios también se pueden encontrar pequeñas fibras musculares lisas intimales, dispuestas longitudinalmente, las cuales expresan SMA en su escaso citoplasma.

A medida que va avanzando la enfermedad varicosa, la lámina íntima de las venas comienza a experimentar un engrosamiento y la estructura y composición de esta sufre cambios importantes y, en principio no reversibles. En los segmentos con una patología más inicial, es decir, en aquellos con una altura de la íntima de 40 µm o menor, la proporción de área que ocupa el colágeno tipo I, principal colágeno estructural del tejido conjuntivo intimal junto al colágeno III,[299] respecto es al

área total de la íntima es de 0,24. Pero a medida que aumenta el engrosamiento intimal, la cantidad de colágeno tipo I relativa aumenta, al principio muy rápidamente (grupo de altura intimal entre 40 y 90 µm) hasta alcanzar una proporción 0,42 y luego en los segmentos más comprometidos se estabiliza en torno al 0,35. Es interesante comparar estos datos con lo que le sucede al otro colágeno más abundante en las venas, el colágeno tipo III.[299] Observamos como en condiciones sin casi patología o una patología inicial, la proporción de área que ocupa el colágeno es de 0,42, manteniéndose en el siguiente grupo de altura en el mismo rango (proporción 0,43), pero disminuye en aquellos casos con una lesión bien establecida.

Es interesante destacar que, si bien ambos tipos de colágeno se expresan en toda la altura de la íntima, es mucho más evidente en la porción superficial e intermedia de los casos con evidente engrosamiento intimal. Además, la expresión de colágeno III es muy intensa en el tercio más superficial del engrosamiento intimal, formando una banda de tejido fibroso homogéneo y muy denso de colágeno III sobre la que se apoya el endotelio venoso (figura 18 y 19), lo que impide un correcto intercambio de metabolitos en la pared venosa varicosa. Sin duda, estas alteraciones determinan cambios hemodinámicos irreversibles del flujo venoso en los pacientes con IVC.[240]

Asimismo, se cuantificaron los niveles de expresión de mRNA para el colágeno tipo I y el mRNA el colágeno tipo III, con el fin de obtener datos moleculares de la patología varicosa de las venas. Se comparó la expresión de los dos colágenos en toda la pared vascular entre el segmento distal y el proximal, ante la imposibilidad física de separar la íntima venosa de la túnica media. Esta limitación nos impide una comparación o correlación directa con los datos histomorfometricos de las lesiones intimales. Pero sí nos permite una inferencia de la posible patogénesis de la enfermedad varicosa. Nuestros datos moleculares mostraron, al comparar la expresión de mRNA de colágeno tipo I y colágeno tipo III, una sobreexpresión de ambos tipos de colágenos en el segmento proximal, el más dañado en la enfermedad varicosa, con respecto al segmento distal.

Se sabe que la proporción de los diferentes tipos de colágenos estructurales (sobre todo colágeno I y colágeno III) varían de un órgano a otro y, sobre todo, cambian sustancialmente en situaciones patológicas relacionas con mecanismos de fibrosis y reparación tisular.[136] En las venas varicosas estudiadas, si comparamos los dos colágenos entre sí, los datos morfométricos obtenidos nos demuestran una progresiva modificación de la ECM de la íntima vascular, sustituyéndose el colágeno III por el colágeno I. Estos datos coindicen, en parte, con los obtenidos

por Xu J. *et al.* 2014[299], quienes también encuentran una disminución de colágeno III en la íntima, evaluada mediante técnicas histológicas. Además, el aumento de la expresión de mRNA de colágeno I que observamos en nuestro material es refrendado por el grupo de Sansilvestri-Morel P. *et al.* 2001[241]. Otros autores también describen un incremento en los niveles de mRNA de colágeno I, pero no encuentran diferencias en los niveles de colágeno III.[121]

En este sentido, la actividad de síntesis de colágeno en la íntima venosa es distinta, dependiendo del número y de la distribución de los fibroblastos y de los miofibroblastos asociados a las áreas de fibrosis intimal. Estudios de cultivos de fibroblastos en rata han encontrado que, el colágeno I y el colágeno III inducen la proliferación de fibroblastos, mientras que el colágeno IV induce la desdiferenciación de los fibroblastos en miofibroblastos.[200] También, en el estudio ultraestructural que hemos realizado describimos la presencia en la íntima de un deposito amorfo de ECM y finos fascículos de colágeno y una irregular distribución de células fusiformes que corresponden a fibroblastos y miofibroblastos. Los fibroblastos tienen un núcleo electrodenso y un citoplasma con abundantes filamentos intermedios de vimentina; mientras que, los miofibroblastos presentan pequeñas vesículas en vez de caveolas (lo que los diferencia de verdaderos leiomiocitos) y abundantes prolongaciones del citoplasma que contienen numerosos miofilamentos relacionados con cuerpos densos y placas de anclaje, pero carecen de conos sarcoplásmicos. Todos estos hallazgos ultraestructurales son característicos de los miofibroblastos. Además, el colágeno I, pero no el colágeno III, activaría la vía ERK1/2 en fibroblastos.[62] De todas formas, según nuestros datos histológicos, la disminución de colágeno III y el aumento de colágeno I provocarían una disminución del ratio de colágeno III respecto al colágeno I, lo cual que afectaría a determinaría un comportamiento distinto de la respuesta en la pared venosas de la resistencia a la tensión. Esta sustitución de colágeno III, que contribuye a la elasticidad, por colágeno I, un colágeno más duro, provocaría mayor fibrosis y una mayor rigidez en la vena varicosa. Como previamente han señalado Fan CM 2005[76], la síntesis anormal de colágeno provocaría un debilitamiento y expansión de la base de las válvulas venosas, lo que muy probablemente determinaría una aposición incompleta de estas y, por tanto, el consiguiente reflujo, aunque las válvulas no tuvieran un especial daño. Está claro, pues, que todos estos mecanismos apoyarían la hipótesis de la debilidad primaria de la pared venosa, frente a la hipótesis valvular.

En la primera fase de engrosamiento intimal, tanto en los segmentos distales, como en los proximales, se observa un progresivo incremento de SMCs SMA+, las cuales se disponen en fascículos longitudinales, situados por dentro de la lámina elástica interna. En el segmento proximal, cuando el engrosamiento intimal es moderado, la mayor parte de la superficie de la íntima está ocupada por células SMA+, las cuales muestran signos de hipertrofia. Sin embargo, a medida que se va incrementándose el engrosamiento intimal, se observan SMCs atróficas localizadas cerca del endotelio; mientras que otras SMCs son hipertróficas y se disponen en posición más basal. Asociados a estos cambios que experimentan los leiomiocitos de la íntima, progresivamente se va incrementando el área ocupada por colágeno I y III, siendo más abúndate el colágeno I en la zona más superficial de la íntima muy engrosada, formando una zona de tejido fibroso más compacto debajo del endotelio vascular, tal como previamente hemos comentando, produciéndose rugosidades irregulares de la superficie endovascular de la vena y modificando las características del flujo sanguíneo circulante, tal como han demostrado estudios hemodinámicos previos en vena safenas varicosas.[199]

En estos segmentos venosos con mínimos cambios intímales, nuestros datos morfométricos cuantitativos revelan que la superficie ocupada por todas las SMCs inmunomarcadas con anticuerpo anti-SMA es baja, si se compara con la superficie total intimal, evaluada en cada campo microscópico. Sin embargo, en este estadio más inicial de lesiones secundarias a IVC, cuando los datos se expresan en valores relativos del área ocupada por las SMCs en relación con el área total intimal, se demuestra que aproximadamente el 29% del engrosamiento intimal corresponde a SMCs; mientras que el resto de la superficie intimal está ocupada por el depósito de finas láminas elásticas, de material elástico granular y amorfo, y por la ECM virtual subendotelial. Contrariamente, histológicamente hemos encontrado que en los casos de lesiones varicosas completamente establecidas y con evidente engrosamiento intimal, se aprecia una importante heterogeneidad del tamaño, orientación y densidad de las SMCs presentes en la íntima vascular, de modo que, existen áreas en las que se ven abundantes leiomiocitos hipertróficos, con extensa expresión de SMA, y dispuestos longitudinalmente, hasta otros campos microscópicos en los que las SMCs SMA+ son menos abundantes, tienen escaso citoplasma y están rodeadas por amplias áreas de la ECM del tejido conjuntivo. Asimismo, el estudio morfológico de los cortes semifinos y ultrafinos de la pared venosa varicosa y las células contenidas en ella realizado, nos permitió

analizar con más profundidad los cambios de la íntima vascular. Es evidente en los cortes semifinos que las células observadas presentes en la íntima presentan en la porción más apical de la misma un núcleo redondeado, escaso citoplasma y están aisladas entre si. Mientras que, las células intimales situadas en la porción más basal presentan un núcleo con forma de huso y menor cantidad de citoplasma. Además, a medida que se engruesa la íntima, la capa de ECs empieza a desaparecer, y aparecen células con núcleos hipertróficos y escaso citoplasma. Asimismo, las SMCs localizadas en la íntima sufren desdiferenciación como señalan su morfología más estrellada en lugar de su forma alargada más habitual.

En definitiva, a medida que la lesión varicosa evoluciona y la íntima se engrosa, se produce una proliferación, en términos absolutos, de SMCs, aunque la proporción del área intimal ocupada por la expresión SMA en la íntima venosa decrece progresivamente según crece la íntima hasta alcanzar un área relativa del 16%.

Las fibras elásticas de la íntima también experimentan cambios muy importantes en relación con el progresivo engrosamiento intimal de los segmentos venosos con lesiones varicosas. En nuestro conocimiento, después de una amplia revisión bibliográfica no hemos encontrado estudios previos que específicamente se centren en las posibles alteraciones de las fibras elásticas, tanto de la lámina elástica interna como de las fibras elásticas presentes en la ECM de la capa íntima. Los datos histológicos obtenidos en la presente Tesis demuestran que el incremento del material elásticos es muy evidente en la capa íntima, pero no se forman verdaderas fibras elásticas, si no que el material elástico depositado es muy irregular, asociándose muchas veces la rotura y desaparición de la lámina elástica interna de la pared venosa. El significado histofisiológico de estos hallazgos acerca de la degeneración de las fibras elásticas de la íntima de venas varicosas son difíciles de interpretar, dado que en venas no existen publicaciones previas, y todo lo que se sabe de alteración de fibras elásticas vasculares se ha obtenido de su estudio en el territorio arterial, cuyas condiciones hemodinámicas y de tono vascular son bien diferentes al lecho venoso.[147,306] Está claro que las fibras elásticas han sido ampliamente estudiadas en el sistema vascular arterial normal, sobre todo en segmentos de aorta y arterias musculares en pacientes con arteriosclerosis[5,85] y también en las arterias de fino calibre y arteriolas que constituyen la microcirculación o en pacientes con diabetes mellitus[222] en los que se han encontrado alteraciones de los mecanismos de remodelación de las fibras elásticas.

En relación con los mecanismos de elastogénesis, se ha demostrado que, en la pared arterial y también en la pared venosa, las fibras musculares lisas tienen la capacidad de sintetizar fibras elásticas;[14] por ello, la elastogénesis observada en las lesiones intímales de venas varicosas es un proceso que, en las fases más iniciales, suele relacionarse con la hipertrofia de fibras musculares lisas intímales, tal como se ha demostrado en el presente trabajo. Sin duda, las alteraciones de las fibras elásticas de la íntima venosa son muy importantes, ya que lo que se observa son fibras elásticas muy cortas y delgadas, asociadas a un depósito difuso de material elástico amorfo. Estos cambios que experimentan las fibras elásticas en los segmentos venosos contrastan con los las lesiones descritas previamente de la fibras elásticas de las arterias musculares de pequeño calibre en modelos exprímeteles de hipertensión arterial, en los que las láminas elásticas permanecen circunferenciales concéntricas, pero lo que se incrementa es el tamaño de las fenestras.[13] De hecho, en las arterias de resistencia, esto es, en arterias musculares finas y arteriolas de pacientes o de modelos experiméntales de hipertensión, se han demostrado lesiones degenerativas irreversibles de las láminas elásticas de su pared.[15] Estos hallazgos morfológicos de la fibras elásticas en estas arterias coinciden, en parte, con lo que también hemos encontrado en la íntima engrosada de venas con varices, en las que se ve un depósito de material elástico y fibras cortas, tortuosas y de fino calibre.

Estos cambios de elastólisis de la lámina elástica interna a nivel de las lesiones de engrosamiento intimal varicoso y sobre todo el deposito de material elástico degenerando y de fibras entrecortadas y finas embebidas en la ECM amorfa de la íntima son lesiones histológicas bastante semejantes a los observadas en otros órganos durante los procesos degenerativos de las fibras elásticas a medida que avanza la edad.[292,295] En efecto, en pacientes de mediana edad, en los que la IVC es frecuente, y sobre todo en la senectud aparecen cambios de la organización de las fibras elásticas de múltiples órganos (piel, vasos sanguíneos, tejido elástico asociado al músculo visceral, etc.), provocándose una elastólisis progresiva, caracterizada ultraestructuralmente por una degeneración del centro claro de elastina, la cual puede contener depósito de material electrodenso muy irregular, y evidente disminución del manto periférico de fibrillas.[210] Estos cambios determinan el entrecortamiento de las fibras elásticas, las cuales están rotas, dicotomizadas y frecuentemente están sustituidas por un material elástico de aspecto granular.[210]

Así, los datos de nuestro estudio morfométrico de la superficie de la íntima ocupada por las fibras elásticas demuestran que, a medida que aumenta la altura de la capa íntima la proporción de las fibras elásticas disminuye. En el primer grupo con mínimo engrosamiento intimal, estas fibras elásticas ocupan un 32% del área de la capa íntima, disminuyendo significativamente en el segundo grupo, representando solo el 19% de la superficie intimal. Además, la cantidad de fibras elásticas desciende muy notablemente en el grupo de lesión intensa, con alturas de la íntima superiores a las 90 µm; en este caso los datos morfométricos que hemos obtenido demuestran que las fibras elásticas solo representan un 13%. Aun así, en estos casos de importante engrosamiento intimal, las fibras elásticas sintetizadas *de novo* están muy desorganizadas y no forman láminas circunferenciales, sino que se ven pequeños discos de fibras de elastina y un material amorfo distribuido irregularmente por la ECM. De hecho, el grupo de Xu N. *et al.* 2014,[300] utilizando una tinción de van-Gieson orceína, una técnica histológica similar a la usada por nosotros, encuentran una obvia disminución, fragmentación y desestructuración de las fibras elásticas en venas varicosas.

En la presenta Tesis hemos efectuado un estudio mediante microscopia confocal de las lesiones intímales de la vena, con el fin de evaluar la disposición y relación entre las fibras elásticas y las fibras de colágeno. Para ello, hemos obtenido pequeños segmentos de la porción proximal y distal de la vena, los cuales han sido fijados *in toto* orientando la superficie endovascular, que es el primer estrato sobre el que incidió el rayo láser del microscopio confocal. En nuestro conocimiento, esta metodología no ha sido previamente usada en otros estudios de la histopatología venosa. Nuestros resultados muestran una íntima relación entre el material elástico depositado en la íntima engrosada y las fibras de colágeno y la ECM. Cuando se evalúa un único plano, mediante microscopia confocal, se demuestra que la fibras de colágeno son muy cortas y delgadas y el material elástico aparece como un deposito granular amorfo y más infrecuentemente como pequeñas fibras elásticas autorefringentes. Estos hallazgos, en parte, son semejantes a las áreas de fibrosis intimal en modelos experimentales de arterioesclerosis, evaluados también *in toto* por microscopia confocal; sin embargo, en estos modelos de ateroesclerosis, las placas de ateroma contienen lípidos y células inflamatorias.[236]

Para comprender mejor los mecanismos íntimos que experimenta la pared venosa ya desde los primeros estadios del desarrollo de la enfermedad varicosa es conveniente cotejar los cambios

hemodinámicos con las lesiones histológicas de la íntima descritas en el presente estudio. Resulta verosímil suponer que cronobiológicamente lo primero que se produzca sean pequeños cambios hemodinámicos, pequeñas turbulencias del flujo venosos en determinados puntos de la superficie endovascular, como consecuencia de las lesiones de engrosamiento mínimo intimal; estas pequeñas lesiones iniciales también se pueden observar en las zonas de la íntima en donde se implanta la base de las valvas de las numerosas válvulas que tiene la vena safena interna. Este reflujo venoso, así como el éstasis sanguíneo asociado, determinan un incremento de la presión hidrostática que, a su vez, determina un aumento de la incompetencia de las válvulas venosas.

Como respuesta a estos cambios funcionales, en los primeros momentos del desarrollo de la enfermedad varicosa las SMCs de la pared experimentan un aumento del tono vascular, lo cual va acompañado de una hiperplasia de SMCs en la íntima vascular, aunque la proporción de SMA disminuya según aumenta la altura de la capa íntima. Esta hiperplasia celular en la túnica íntima, como ya hemos dicho, se acompaña de un incremento también progresivo de la superficie ocupada por colágenos I y III, aumentando la proporción de colágeno I según aumenta la altura, mientras que el colágeno III aumenta en un principio para luego estabilizarse. Además, aparece una disminución en la proporción de por fibras elásticas a medida que aumenta la íntima fibrosada. Esta correlación morfométrica que hemos encontrado cuando evaluamos histológicamente la expresión de diferentes moléculas en la íntima varicosa es lógica, ya que se sabe que las SMCs son capaces de sintetizar colágenos, degradar elastina y participar en los mecanismos de elastogénesis y remodelado vascular.[36,129,287]

Además, estos datos están en consonancia con estudios *in vitro* que han encontrado que las SMCs derivadas de venas safenas varicosas están más desdiferenciadas y muestran un aumento de la capacidad proliferativa y de síntesis de proteínas, en comparación con la observada en las SMCs de venas normales, pasando de un fenotipo contráctil a uno secretor.[164] Se ha sugerido que estos mecanismos pudieran contribuir a la remodelación de la pared venosa y al debilitamiento de su capacidad funcional, debido al estrés mecánico que sufre la vena safena en pacientes con IVC.[74,199] A pesar de los estudios realizados centrados en mecanismos implicados en proliferación en venas safenas,[16,71] no se conocen los mecanismos íntimos de esta proliferación y diferenciación de las SMCs intímales.[35,50,93,97]

Estudios recientes han demostrado la participación del endotelio vascular en la regulación de mecanismos de estrés oxidativo frente a alteraciones locales del flujo hemodinámico y de la presión intraluminal, los cuales pueden modificar la composición de la pared venosa.[147,306] Moléculas ROS relacionadas con el estrés oxidativo es posible que pudieran regular la proliferación y diferenciación de células intímales de la pared venosa, las cuales se transformarían en miofibroblastos y SMCs, presentes en las fases iniciales de la fibrosis intimal varicosa, tal como hemos demostrado inmunohistoquímica y ultraestructuralmente en la presente Tesis. Pero también cabe especular que la presencia de SMCs SMA+ encontradas en la íntima engrosada procedieran en parte de la migración de los leiomiocitos de la túnica media vascular.

Está establecido que las fuerzas biomecánicas son, de una parte, la fuerza de fricción de la sangre que actúa sobre el endotelio y, de otra, la tensión circunferencial que actúa tanto sobre el endotelio, como sobre las SMCs de la íntima y de la media y sobre los fibroblastos de la adventicia CITAS. Estas fuerzas biomecánicas inducen el inicio de mecanismos de inflamación, como consecuencia de la interacción entre el endotelio y los leucocitos circulantes. Es más, estas modificaciones del tono vascular producen cambios de señalización de las ECs y liberación de moléculas bioactivas hacia el tejido conjuntivo intimal.[190,199] Sin duda, estos cambios funcionales que ejercen modificaciones iniciales del flujo y de la presión intraluminal posiblemente sean los que desencadenan los primeros cambios de la ECM de la íntima venosa (tal como se ha demostrado en la íntima en modelos de hipertensión arterial,[156,157] y subsecuentemente el inicio del incremento del espesor intimal y la diferenciación de SMCs SMA+. Por ello, ya desde estas fases iniciales subclínicas de IVC se produce una progresiva desestructuración morfológica y una modificación molecular de los segmentos de pared venosa con lesiones varicosas.[147,306]

Es posible que mínimos cambios hemodinámicos locales, derivados del éstasis venoso y de la hipertensión secundaria, sean suficientes para provocar un estrés en la pared de la vena safena varicosa, lo cual pudieran determinar cambios fisiopatológicos que den lugar al inicio de mecanismos de remodelación de la túnica íntima. Se sabe que estos cambios hemodinámicos dan lugar a unas fuerzas biomecánicas determinadas por el flujo sanguíneo, la presión intra y extraluminal y las diferentes cargas tensiónales que actúan en cada segmento venoso comprendido entre dos válvulas; todos estos mecanismos son los responsables de que las lesiones varicosas a lo largo de la misma vena sean multifocales y de diferente intensidad.[274] La exposición

a altas presiones en el extremo inferior de la pierna provocaría cambios en la propiedades estructurales y venosas que conlleva todo un remodelamiento vascular. En este remodelamiento vascular está implicada la migración, proliferación y apoptosis de ECs y SMCs, así como la síntesis y degradación de ECM.[156]

Por otro lado, en la formación de la neoíntima, tanto en las patologías arteriales y venosas primarias y también en los injertos vasculares autólogos,[230] intervienen procesos de óxido reducción, dando lugar a una activación de mecanismos enzimáticos que regulan facilitando o inhibiendo la formación de dicha neoíntima.[230] Las alteraciones de la pared venosa en pacientes con IVC pueden estar desencadenadas por mecanismos de estrés oxidativo, los cuales, tal como se ha demostrado en arterias de elevada resistencia, determinarían alteraciones estructurales y moleculares de las fibras elásticas, que dificultarían los mecanismos de reparación y de remodelación vascular.[56]

En relación con el estrés oxidativo, las ECs participan en la regulación de ROS y de este modo participan en los mecanismos de tipo inflamatorio que experimentan las ECs y las SMCs de la pared vascular.[106] Está demostrado que en los pacientes con IVC se produce un estado de hipertensión vascular venosa, secundario al estasis venoso, lo cual puede desencadenar mecanismos proinflamatorios y liberación de interleucinas, tal y como se ha señalado en otras situaciones de alteraciones hemodinámicas vasculares que cursan con hipertensión.[6] Por tanto, el endotelio experimenta cambios funcionales y estructurales secundarios al estrés oxidativo y al proceso inflamatorio.

Estas alteraciones endoteliales han sido ampliamente estudiadas en las fases iniciales de las lesiones ateroscleróticas y también en los mecanismos de endotelización de la neoíntima presente en los injertos vasculares;[294] así mismo, en modelos experimentales de hipertensión venosa crónica (hipertensión portal) se ha demostrado el desencadenamiento de un estrés oxidativo de la pared vascular.[65]

En nuestro conocimiento la pormenorizada revisión de la literatura que realizamos en el momento de comenzar la presente Tesis no existían estudios previos que compararan las alteraciones histológicas y cuantitativas de la íntima de las venas varicosa con métodos moleculares que exploraran mecanismos de estrés oxidativo y de inflamación. Por ello, creemos que el planteamiento de objetivos de la presente Tesis en los que se cotejan los hallazgos

histológicos con datos moleculares de estrés oxidativo y de mecanismos inmunológicos aporta nuevos conocimientos en la histofisiología de las lesiones iniciales de la enfermedad varicosa. De hecho, el éstasis venoso en estos pacientes, ya desde los primeros estadios del desarrollo de IVC, produce daño endotelial, aumento de ROS y liberación de moléculas proinflamatorias, las cuales tienen la capacidad de atraer hacia la pared venosa lesionada monocitos y macrófagos, capaces de degradar la ECM.

En nuestro estudio hemos encontrado una significativa mayor expresión de mRNA de NOX-4, así como una mayor producción de H_2O_2 y, asimismo, una significativa mayor actividad NADPH oxidasa en el segmento venoso proximal, con evidentes lesiones intimales de varices, con respecto al segmento distal de la vena safena, que habitualmente presenta mínimas lesiones. Sin embargo, nos encontramos expresión de mRNA de NOX-1 en las muestras de venas safenas que hemos estudiado. En estudios realizados en arterias, de las 7 isoformas de NOX conocidas, se expresan NOX1, NOX2, NOX4 y NOX5.[37,48] La expresión de NOX-4 es significativamente mayor que las otras isoformas en arterias.[37,191] Con todo, consideramos que nuestros datos de mRNA de NOX-4, producción de H_2O_2 y actividad NADPH oxidasa son de gran interés, ya que la sobreexpresión en el segmento proximal de NOX-4, una de las principales enzimas implicadas en la producción de H_2O_2, se correlaciona con una mayor producción de H_2O_2 y con una significativa mayor actividad NADPH oxidasa, lo que sugiere un mayor estrés oxidativo en el segmento proximal que en el segmento distal de las venas varicosas. En este sentido, estudios previos han demostrado que los pacientes con IVC padecen un incremento en el estrés oxidativo,[44,140] aunque no lo relacionan con el grado de alteraciones de la pared venosa. Estos mecanismos sugeridos en la regulación de la fibrosis progresiva de la íntima venosa pueden ser verosímiles, dado que está comprobado que la activación de NADPH oxidasa incrementa la producción de ROS procedente de las mitocondrias, y viceversa, que las ROS procedentes de la mitocondria son capaces de activar NADPH oxidasa, especialmente en condiciones patológicas;[67] además, las ROS mitocondriales y la NADPH oxidasa participan en la regulación de la expresión y actividad de la xantina oxidasa[247] y el desacoplamiento de eNOS en las ECs.[247] También el H_2O_2 es capaz de activar la NADPH oxidasa y provocar un aumento de la producción de O_2^-, además, el H_2O_2 es capaz de inducir la transición de la xantina deshidrogenasa en xantina oxidasa, que es capaz de generar tanto H_2O_2 como $O_2^{-\cdot}$,[185] lo cual determina aún un mayor daño celular.[82]

Se sabe que la enzima COX-2 responde a estímulos pro-inflamatorios catalizando el primer paso de la biosíntesis de prostanoides: prostaglandinas y tromboxano, siendo los macrófagos y ECs la fuente principal de las dos isoformas de COX en la IVC.[1] En los tejidos de la pared venosa varicosa que hemos evaluados encontramos que COX-2 está significativamente sobreexpresada en el segmento proximal venoso, también la expresión de mRNA de m-PGES-1 tiende a ser mayor en el segmento proximal que en el segmento distal de las venas estudiadas, aunque el análisis estadístico no encontró diferencias significativas. Estudios recientes de otros autores han establecido que la forma inducible de la ciclooxigenasa es tradicionalmente considerada como un biomarcador temprano de inflamación.[238] Además, la actividad de la peroxidasa de la COX cataliza la generación de un producto intermedio inestable, la prostaglandina (PG)H_2. Una prostaglandina sintasa especifica, la mPGES, convierte PGH_2 a PGE_2, que es la más abundante prostaglandina pro-inflamatoria asociada en condiciones inflamatorias. Algunos estudios han demostrado el papel de PGE_2 en la expresión/activación de metaloproteinasas en tejidos humanos, fundamentalmente en el estudio de SMCs provenientes de aorta,[304] pero en nuestro conocimiento no han sido estudiadas en la pared venosa varicosa.

Al igual que en nuestro estudio, el grupo de Bertrand-Thiebault C. *et al.* 2004[29] identifica la expresión de COX-2 mRNA en venas varicosas. Sin embargo, en una publicación reciente se ha señalado la ausencia de COX-2 en venas varicosas.[95] Aunque la expresión de COX-2 ha sido considerada un importante factor de la citotoxicidad asociada con inflamación,[250] los factores responsables de la citotoxicidad de COX-2 no han sido completamente definidos. En el daño tisular presente en las venas varicosas, podría estar implicada la producción de prostanoides pro-inflamatorios,[250] de modos que la producción de ROS y su relación con mecanismos de peroxidación, cuando PGG_2 es convertida en PGH_2,[278] aumentarían los radicales libres que inducirían apoptosis y daño tisular crónico.[107] Estos mecanismos también pudieran estar potenciados por la relación entre COX-2 y las NADPH oxidasas, ya que las NADPH oxidasas pueden ser una fuente de ROS, mediado por la sobrerregulación de COX-2;[160] existiendo además una regulación recíproca entre proteínas NOX y COX-2.[237]

Teniendo en cuenta nuestros resultados, el desencadenamiento de mecanismos pro-inflamatorios, el aumento de ROS y la mayor expresión de COX-2 podrían explicar la sobreexpresión que encontramos de MAC-3 (una proteína presente en el fagosoma de

macrófagos[251] en el segmento proximal de las venas varicosas. Nuestros datos están en consonancia con estudios previos que han descrito la presencia de infiltrados de macrófagos en las válvulas de venas varicosas en la pared de venas varicosas.[244]

Una vez que se desencadenan estos mecanismos oxidativos e inmunológicos en la pared venosa, secundarios al éstasis sanguíneo, comienza el incremento de la ECM en la íntima vascular, tal como hemos demostrado en la presente Tesis, dando origen a una hiperplasia de SMCs y a una progresiva fibrosis intimal con depósito de fibras elásticas y con incremento de colágeno tipo I y colágeno tipo III mucho mayor en la íntima del segmento proximal que del distal. Como previamente se ha comentado, todos estos hallazgos histológicos han sido corroborados en nuestro estudio *in toto* de las lesiones endovasculares realizado mediante microscopia confocal.

La expresión de mRNA de SMA, elastina, colágeno I y colágeno III está sobreexpresado en los segmentos proximales, donde la lesión es más importante y existe una proliferación de SMCs en la capa íntima. Estos resultados coinciden con las observaciones realizadas por el grupo Cario-Toumaniantz C. *et al*. 2007[44], que mediante RT-PCR también encuentran un aumento en la expresión de colágeno I y colágeno III en muestras de tejido de venas varicosas, comparado con venas normales, aunque en cultivos de SMC procedentes de venas varicosas solo el mRNA de colágeno tipo I se encuentra sobreexpresado, mientras que el colágeno III no muestra diferencias. Como es sabido, las SMCs tiene la capacidad de remodelar el colágeno de la matriz extracelular.[281] También el estudio realizado por Lee S. *et al.* 2005[154] muestra sobreexpresión de genes relacionados con moléculas de la ECM. Sin embargo, otros grupos muestran una disminución en el contenido de proteínas colágeno III, mientras que el mRNA de colágeno III no está alterado en cultivos de SMC y fibroblastos dérmicos derivados de pacientes con venas varicosas.[241]

En definitiva, en la presente Tesis se ha demostrado que existen cambios inmunohistoquímicos y cuantitativos de la proporción de SMA, colágeno tipo I, colágeno tipo III y fibras elásticas a medida que aumenta el engrosamiento de la íntima de la vena safena en pacientes con IVC. Estos cambios se asocian también con un incremento de la elastogénesis de la íntima venosa, aunque la superficie ocupada por las fibras elásticas en relación a la superficie total de la íntima disminuye a medida que aumenta el engrosamiento intimal. Todos estos datos sugieren que las lesiones de fibrosis intimal en las venas varicosas son irreversibles cuando progresa el engrosamiento intimal en pacientes con varices.

Nuestros resultados indican que es en las fases iniciales, cuando el engrosamiento es mínimo, el momento de explorar nuevos mecanismos o nuevas moléculas que eviten la progresión de la enfermedad. Hasta el momento presente no se han encontrado tratamientos médicos idóneos para prevenir la IVC y para impedir o remodelar los cambios iniciales intímales, aunque estudios como el nuestro muestran la implicación de mecanismos de estrés oxidativo e inflamación que podrían ayudar en la búsqueda de dianas terapéuticas que eviten el inicio de la lesión varicosa.

Así mismo, es necesario diseñar nuevas investigaciones de correlación de los cambios histopatológicos y de los mecanismos moleculares con estudios hemodinámicos y funcionales de las venas con diferentes grados de enfermedad varicosa; sin embargo, estas correlaciones morfofuncionales han sido ampliamente evaluadas en el territorio arterial. En este sentido, aunque en las arterias se ha estudiado el efecto que tienen las fuerzas biomecánicas producidas por las alteraciones hemodinámicas, aún son pocos los trabajos en venas, pero es sugestivo considerar que el fallo en la respuesta miogénica secundario a alteraciones hemodinámicas tiene un papel importante en el desarrollo de las venas varicosas.

Aun así, este estudio presenta limitaciones potenciales. El estudio histológico de la pared de venas completamente normales en material humano es complicado, dado que la obtención de muestras en pacientes sanos no es éticamente posible. Lo que sí es posible, una vez obtenido el consentimiento informado para investigación, es estudiar segmentos de vena safena procedentes de pacientes sometidos a cirugía coronaria que no son utilizados en el bypass coronario.[122,137] Estos segmentos podrían ser en el futuro una fuente de material del grupo Control para desarrollar nuevos estudios histológicos y moleculares relacionados con los mecanismos de formación de varices. Por último, como material control también podrían ser usados pequeños segmentos de vena aparentemente normal que sobran en la realización de fístulas arterio-venosas, como acceso vascular para los tratamientos de diálisis o aféresis, o de vena safena aparentemente normal no empleada en bypass de extremidades o en revascularización coronaria.[217]

En definitiva, los datos obtenidos en la presente Tesis sugieren que la activación de moléculas relacionadas con mecanismos de estrés oxidativo desencadenan el inicio de procesos proinflamatorios que determinan cambios en la ECM, produciéndose un progresivo aumento de la ECM, con depósito en la íntima de elastina, e incremento del ratio colágeno I/colágeno III , lo que

origina una fibrosis intimal intensa e irreversible que provoca irregularidades en la superficie luminal, con disminución del diámetro de luz. Estas lesiones intímales y de la superficie luminal se podrían correlacionar con las importantes alteraciones hemodinámicas del flujo vascular; de modo que, progresivamente se potenciaría la formación de lesiones varicosas crónicas a todo lo largo de toda la vena safena.

CONCLUSIONS

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FIRST. The saphenous varicose vein shows a progressive intimal thickening with moderate muscular atrophy at the medial layer. The intima presents a collagen I and collagen III deposition and disorganized elastic fibers. Also, SMA, elastin, collagen I and collagen III mRNA are upregulated at the proximal segment respect to the distal segment. However, when the intimal rises, the proportion of collagen I rises too, but the proportion of collagen III, SMA and elastic fibers decrease.

SECOND. The intimal layer from varicose veins has SMA positive SMCs and others ellipsoidal shape cells that are vimentin and SMA positive and their ultrastructure correspond to myofibroblast.

THIRD. Intimal fibrosis studied *in toto* by confocal microscopy show an amorphous elastic material deposition and thin elastic fibers surrender by collagen fibers.

FOURTH. The proximal segment has a higher oxidative stress than the distal segment. The proximal segment has a higher H_2O_2 production, NADPH oxidase activity and NOX-4 mRNA is upregulated.

FIFTH. Our data demonstrated the develop of stress oxidative mechanism in relation to the fibrosis of the intimal layer of varicose saphenous vein. The progressive deposit of collagen and elastic fibers determinate an intense fibrosis of the intimal surface.

CONCLUSIONES

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PRIMERA. La vena safena varicosa presenta un aumento progresivo de la túnica íntima con moderada atrofia de la capa muscular media. En la íntima se observa el depósito de colágeno I, colágeno III y de fibras elásticas desorganizadas. La cuantificación de mRNA muestra un aumento de SMA; elastina, colágeno I y colágeno III en el extremo venoso proximal respecto al distal. Además, morfometricamente se demuestra que, a medida que aumenta la superficie intimal, también aumenta la proporción de colágeno I, aunque disminuye la proporción de colágeno III, SMA y fibras elásticas.

SEGUNDA. En la lesión varicosa intimal se identifican SMCs SMA positivas y acúmulos de células fusiformes vimentina y SMA positivas, que ultraestructuralmente corresponden a miofibroblastos.

TERCERA. La lesión de fibrosis intimal se ha estudiado *in toto* mediante microscopia confocal evidenciándose una estrecha relación entre el depósito de material elástico amorfo y pequeñas fibras elásticas rodeadas por fibras de colágeno.

CUARTA. Existe un mayor estrés oxidativo en el extremo proximal que en el distal, evidenciándose una significativa mayor producción de H_2O_2, un incremento de la actividad NADPH oxidasa y la sobreexpresión de NOX-4.

QUINTA. Nuestras datos demuestran el desarrollo de mecanismos de estress oxidativo en relación con fibrosis de la capa íntima de las venas safenas varicosas. El depósito progresivo de fibras de colágeno y elásticas determian una fibrosis intensa de la superficie intimal.

RESUMEN

La Insuficiencia Venosa Crónica (IVC) de las extremidades inferiores es una enfermedad muy frecuente que causa una gran morbilidad en personas de mediana y avanzada edad. Los mecanismos fisiopatológicos y los datos hemodínamicos de esta enfermedad están bien establecidos. También se conocen las lesiones generales de fibrosis de la capa íntima y de la capa media de las venas con varices. Sin embargo, la histogénesis y los mecanismos de progresión de las lesiones de varices no están completamente establecidos y tampoco se han evaluado pormenorizadamente las alteraciones morfométricas, celulares y moleculares a nivel del segmento proximal (con evidente lesión varicosa) y del segmento distal (con menor lesión varicosa).

En la presente Tesis realizamos un estudio morfométrico de la expresión inmunohistoquímica de alfa actina muscular (SMA), colágeno tipo I, colágeno tipo III y de fibras elásticas en los diferentes grados de engrosamiento intimal. Asimismo, se ha realizado una valoración de segmentos de vena estudiados *in toto* mediante microscopia confocal, con el fin de establecer las relaciones de las fibras elásticas y del colágeno en la porción superficial de la íntima lesionada, método que no ha sido aplicado previamente en la investigación de venas. También se ha realizado un estudio ultraestructural para valorar la matriz extracelular (ECM) y la proliferación celular de la íntima lesionada. Por último se ha estudiado la diferente expresión génica de moléculas implicadas en procesos de inflamación, ROS y de síntesis de colágeno, tanto en el segmento proximal como en el distal de las venas varicosas.

Se estudiaron 20 venas safenas de pacientes en estadio clínico CEAP 2. Las venas safenas fueron extirpadas e inmediatamente procesadas dentro del quirófano para la realización de los experimentos histopatológicos y moleculares. Se usaron anticuerpos monoclonales para identificar inmunohistoquímicamente SMA, colágeno tipo I, colágeno tipo III y vimentina, así como el uso de tinción de orceina para la detección de fibras elásticas. La cuantificación de estas moléculas se realizó mediante el programa ImageJ. Se obtuvieron pequeños fragmentos para el estudio de microscopia electrónica de transmisión. También pequeños segmentos de la región proximal y distal, después de fijarse, se estudiaron *in toto* por microscopia confocal, identificando la capa endoluminal, para valorar

las fibras elásticas y de colágeno de la porción superficial de la capa íntima. Los métodos moleculares que se realizaron fueron RT-PCR, la medida de la actividad NADPH oxidasa y de la producción de peróxido de hidrogeno mediante Amplex Red.

Nuestros resultados demuestran que el engrosamiento intimal en las áreas con lesión varicosa es progresivo y se debe fundamentalmente al depósito de ECM y colágeno tipo I y III. También existe un depósito de material elástico, pero la presencia de fibras elásticas completas es mínima. En la íntima, hemos identificado células proliferantes vimentina y SMA positivas, y hemos demostrado que ultraestructuralmente corresponden a miofibroblastos.

Los datos de la cuantificación de la superficie inmunomarcada con el anticuerpo anti-colágeno I permiten comprobar que, en un primer momento de engrosamiento intimal, la proporción de colágeno I aumenta en el grupo con lesión intimal varicosa moderada (39,9 a 89,9 µm) y luego se estabiliza en torno al 35% en le grupo con una mayor lesión. La morfometría realizada para cuantificar la superficie intimal ocupada por colágeno III muestra una proporción en torno al 40% en los dos primeros grupos (engrosamiento intimal inicial e intermedio), para descender bruscamente en el tercer grupo de engrosamiento intimal intenso hasta valores en torno al 30%.

El estudio morfométrico de la superficie marcada inmunohistoquímicamente con anticuerpos anti-SMA demuestra que las fibras musculares lisas de la capa íntima aumenta a medida que se produce el engrosamiento intimal. Sin embargo, cuando se calcula la proporción del área SMA+, con respecto a la superficie total de la íntima, se observa una progresiva disminución del músculo liso en los grupos con mayor engrosamiento intimal.

La morfometría de las fibras elásticas orceína positivas muestra que la superficie ocupada por las fibras elásticas va aumentando progresivamente y paralelamente al incremento de la superficie de la íntima. Sin embargo, a pesar del desarrollo de este proceso de elastogénesis intimal, la proporción que ocupan estas fibras elásticas en la íntima, respecto al área total intimal, sufre una progresiva y significativa disminución a medida que aumenta la hiperplasia intimal.

En la presente Tesis hemos evaluado por métodos moleculares las alteraciones existentes tanto en el segmento distal como en el segmento proximal de venas safenas con lesiones varicosas, explorando algunas moléculas implicadas en mecanismos de inflamación y estrés oxidativo. En primer lugar, se cuantifico por RT-PCR la expresión de colágeno tipo I, colágeno tipo III, elastina y SMA. Estos genes están significativamente sobreexpresados en el segmento proximal respecto al distal. También se estudio la expresión de MAC3, COX2, NOX4, NOX1 y mPGES. Nuestros datos demuestran que el mRNA para MAC3, COX2 y NOX4 se encuentra significativamente sobreexpresado en el extremo proximal. Sin embargo, no se encontraron diferencias significativas en el mRNA para mPGES-1 y no se detectó expresión para NOX1 (Figura 32). Por último, se estudió también la diferente producción de peróxido de hidrógeno y la actividad NADPH oxidase, observándose una mayor actividad de ambas moléculas en el extremo de la venas safena con lesiones varicosas.

SUMMARY

The Chronic Venous Insufficiency of the lower limbs (CVI) is a very common disease that causes high morbidity in middle and elderly people. The pathophysiology mechanisms and hemodynamic data are well established. Also, the general fibrosis lesions at the intima and media layer from varicose veins are known. However, the histogenesis and progression mechanisms of varicose veins are not well established. It has been neither evaluated the morphometric, cellular and molecular alterations at proximal segment (with high varicose lesion) compared to distal segment (with small varicose lesion).

In this Thesis, we made a morphometric study of the immunohistochemistry expression of smooth muscle actin (SMA), collagen type I, collagen type III and elastic fibers at the different intimal thickening grades. Additionally, it was done a study *in toto* of vein segments by confocal microscopy, to know better the relation between elastic fibers and collagen fibers at the superficial level of the intima layer. This method has not been done previously at vein research. It was effectuated an ultrastructural study to valuate extracellular matrix (ECM) and cellular proliferation at the intima injured. Last of all, it has been studied the genetic expression of molecules implicated at inflammation, ROS and collagen synthesis, in both proximal and distal segments.

It has been studied 20 saphenous veins from patients at CEAP 2 clinic stage. The veins were removed and immediately were processed inside the surgical room for the realization of the histological and molecular experiments. It has been used monoclonal antibodies to identify SMA, collagen type I, collagen type III and vimentin, and use of orcein stain for elastic fibers detection. The quantification of these molecules was done by ImageJ software. It was also obtained small segments from the distal and proximal fixed vein regions, for confocal microscopy study *in toto*. It was identified the endoluminal surface and it was valuated elastic fibers and collagen fibers at the superficial part of the intimal layer. Moreover, it was done RT-PCR, measure of NADPH oxidase and peroxide hydrogen by Amplex Red for molecular methods in proximal and distal vein samples.

Our results demonstrated that the intimal thickening at areas with varicose lesions is progressive and it due mainly to ECM and collagen type I and III deposits. It also exist an elastic deposition, but the existence of complete elastic fibers is minimum. At the intima, we

have identified proliferative cell that they are positive to vimentin and SMA, that ultrastructurally correspond to myofibroblast.

The data of the immunostain area quantification with antibody anti-collagen type I allows to confirm that in a first moment of intimal thickening, the proportion of collagen type I increase in the group with moderate intimal lesion (39,9 a 89,9 μm) and then it is stabilize around 35% at the group with a higher lesion. The morphometric data about the area occupied by collagen type III has a proportion around 40% at the two first groups (initial and intermediate intimal thickening), but the proportion has an intense decrease to 30% at the group with an intense intimal thickening.

The morphometric study of the total area stain by anti-SMA demonstrate that the muscular fibers from the intima rises when the intima thickening increase. However, if we calculate the proportion of the area occupied by SMA related to the total intima layer, we observe a progressive reduction of the proportion of SMA in the groups with a higher intimal layer.

The morphometric studied by orcein + elastic fibers show that the area occupied by elastic fibers increase when the intimal layer grow. Nevertheless, the proportion of the elastic fibers compared to the total intimal area suffers a progressive and significant decrease when the intima rises.

In this Thesis we have evaluated by molecular methods the alterations presented at the distal and proximal segment from saphenous veins with varicose lesions. We have explored few molecules implicated at mechanism of inflammation and oxidative stress. First of all, we quantify by RT-PCR the expression of collagen type I, collagen type III, elastin and SMA. These genes are significantly upregulated at the proximal segment. We also study the expression of MAC3, COX2, NOX4, NOX1 and mPGES. Our data show that the mRNA for MAC3, COX2 and NOX4 genes are significantly upregulated at the proximal segment. However, we do not find significant difference at the mRNA for mPGES-1 and we do not detected the expression of NOX1. Finally, the different production of hydrogen peroxide and

NADPH oxidase activity were studied. We observed a higher activity of both molecules at the proximal segment from saphenous veins with varicose lesions.

Los datos de la presente Tesis demuestran que las lesiones histológicas de la íntima, ya desde los primeros estadios de la enfermedad varicosa, se caracterizan por un incremento importante del tejido conjuntivo asociado a una proliferación focal de miofibroblastos. En estas áreas, la capa media muscular sufre atrofia de leiomiocitos y fibrosis intersticial. Además nuestros datos moleculares indican alteraciones relacionas con el estrés oxidativo e infamación que desencadenarían el inicio y la progresión de los procesos de engrosamiento y fibrosis intimal.

The data from this Thesis demonstrate that the histological lesions at the intima layer, since the initial stages of the varicose disease, are characterized for an important increase of connective tissue associated to a myofibroblast proliferation. In these areas, the muscular media layer suffers atrophy of leiomyocytes and interstitial fibrosis. Moreover, our molecular data indicated alterations related to oxidative stress and inflammation that develop the beginning and progression of the fibrosis and thickening of the intima.

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